Aplicaciones de la citometría de flujo en el laboratorio de diagnóstico hematológico

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Citometría en Hematología

Banco de Sangre

- ✓ Cuantificación de CD34
- ✓ Control de calidad de hemoderivados (leucocitos)

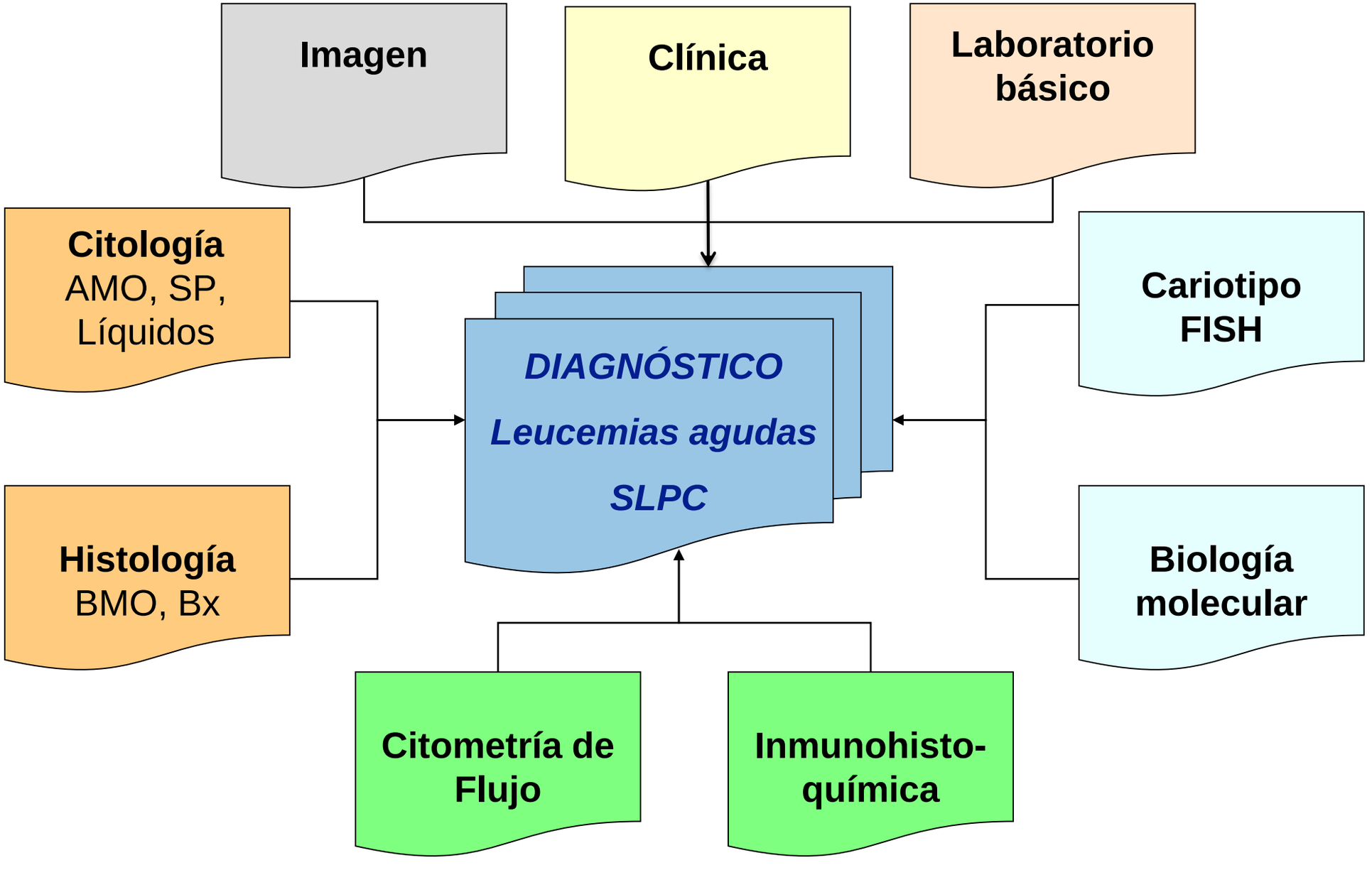
Hemostasia

- ✓ Trombocitopatias hereditarias
- ✓ Activación plaquetar
- ✓ Micropartículas circulantes procoagulantes

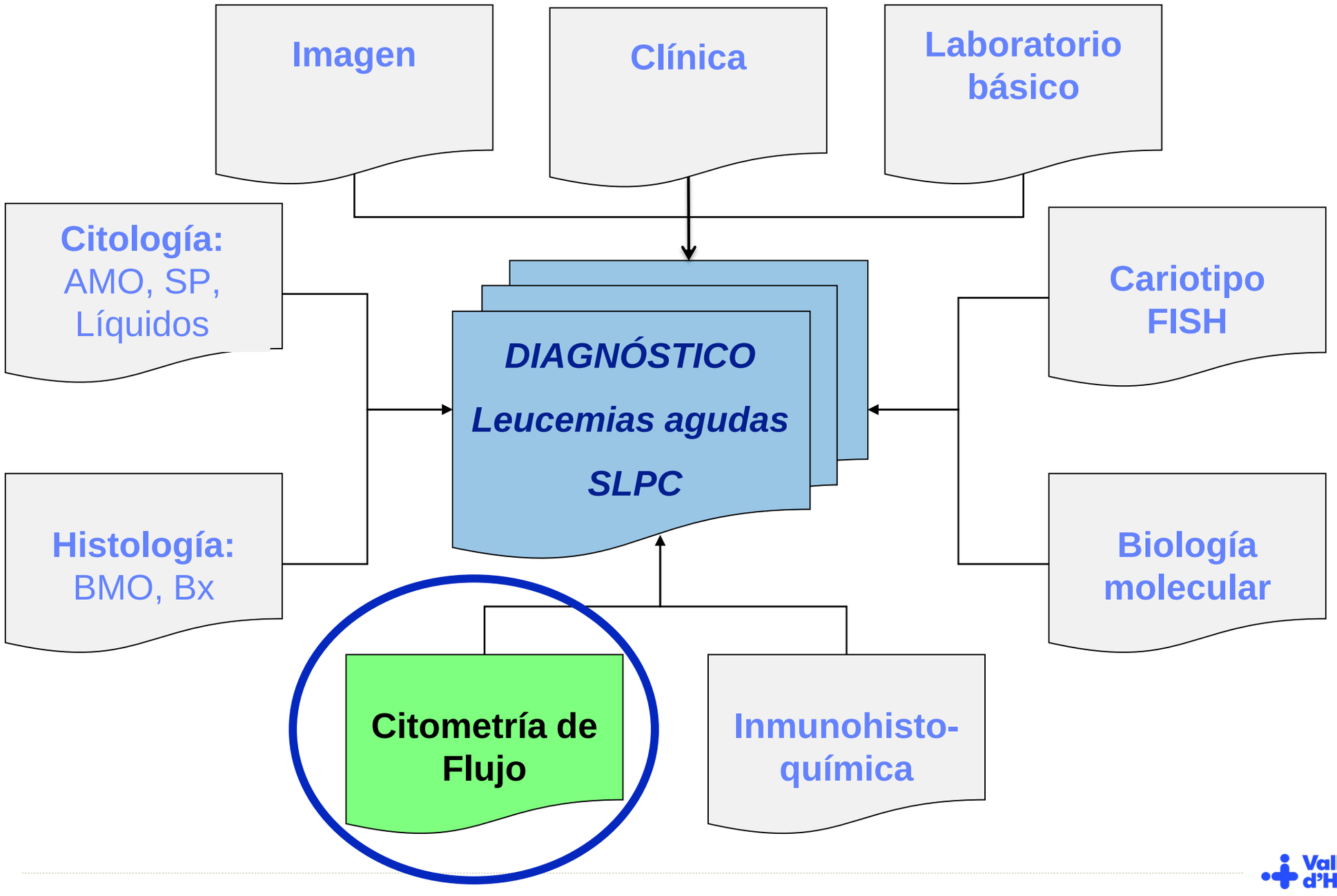
Hematopatología

- ✓ Neoplasias mieloides
- ✓ Neoplasias linfoides

Diagnóstico en Hematopatología



Diagnóstico en Hematopatología



Citometría en Hematología

Indicaciones del estudio de Citometría de Flujo

En el momento del diagnóstico

- ✓ Pancitopenia
- ✓ Leucocitosis (linfocitosis y monocitosis)
- ✓ Observación de células atípicas o blastos
- ✓ Plasmocitosis o gammapatía monoclonal
- ✓ Organomegalias (especialmente adenopatías y esplenomegalia).

Davis BH et al. 2006 Bethesda International Consensus Recommendations on the Flow Cytometric Immunophenotypic Analysis of Hematolymphoid Neoplasia: Medical Indications. Cytometry B Clin Cytom 2007

Citometría en Hematología

Aplicaciones del estudio de Citometría de Flujo

En el momento del diagnóstico

- ✓ Forma parte del diagnóstico
- ✓ Identificación de posibles dianas terapéuticas (CD20, CD52, CD38 …)
- ✓ Identificación de factores pronósticos
- ✓ Estudio de extensión (médula ósea, SNC)
- ✓ Valoración de la celularidad normal acompañante

Davis BH et al. 2006 Bethesda International Consensus Recommendations on the Flow Cytometric Immunophenotypic Analysis of Hematolymphoid Neoplasia: Medical Indications. Cytometry B Clin Cytom 2007

Citometría en Hematología

Aplicaciones del estudio de Citometría de Flujo

Durante el seguimiento

- ✓ Monitorización de la respuesta al tratamiento
  - Enfermedad residual
  - Afectación de SNC
- ✓ Valoración de otras poblaciones celulares (CD4)
- ✓ Identificación de patologías intercurrentes (t-MDS, t-AML, PTLD)
- ✓ Identificación de la progresión o recaída

Davis BH et al. 2006 Bethesda International Consensus Recommendations on the Flow Cytometric Immunophenotypic Analysis of Hematolymphoid Neoplasia: Medical Indications. Cytometry B Clin Cytom 2007

Citometría en Hematología

Muestras para estudio de Citometría de Flujo

No requieren disgregación previa

✓ Sangre

✓ Médula ósea (aspirado medular)

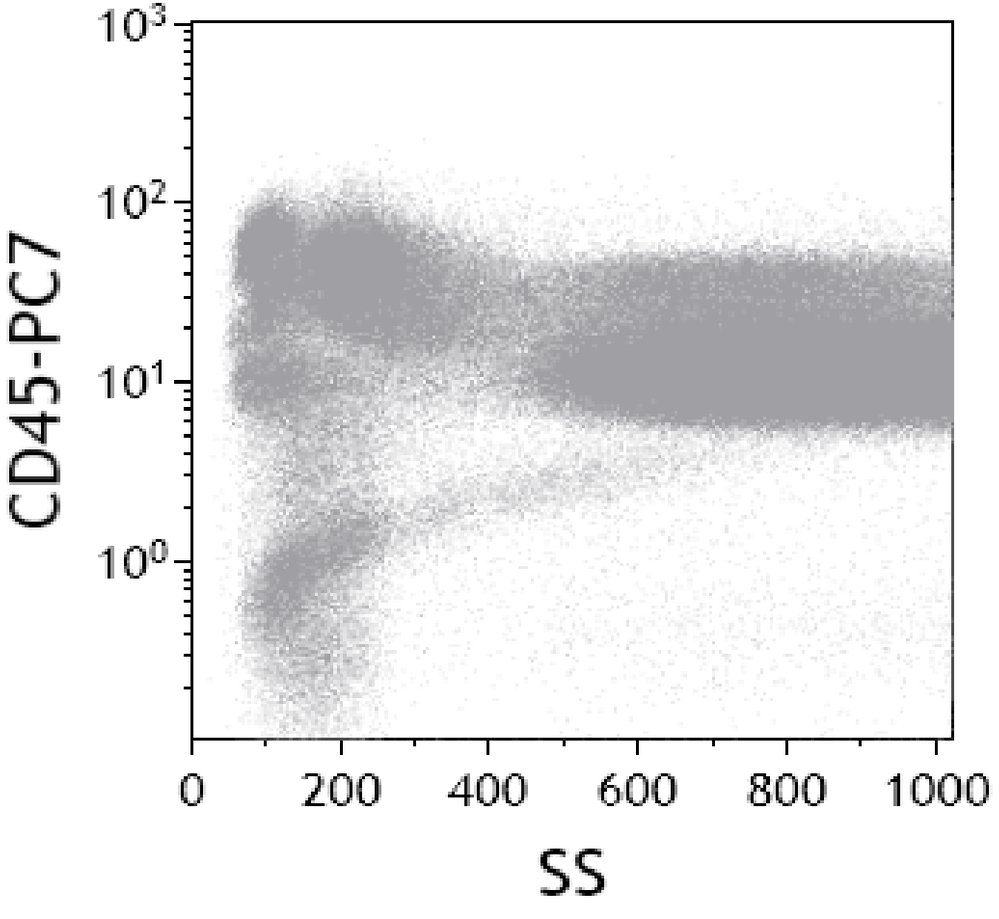
✓ Líquidos orgánicos (ascítico, pleural, LCR, vítreo)

✓ Punción-aspiración con aguja fina (PAAF)

Requieren disgregación previa

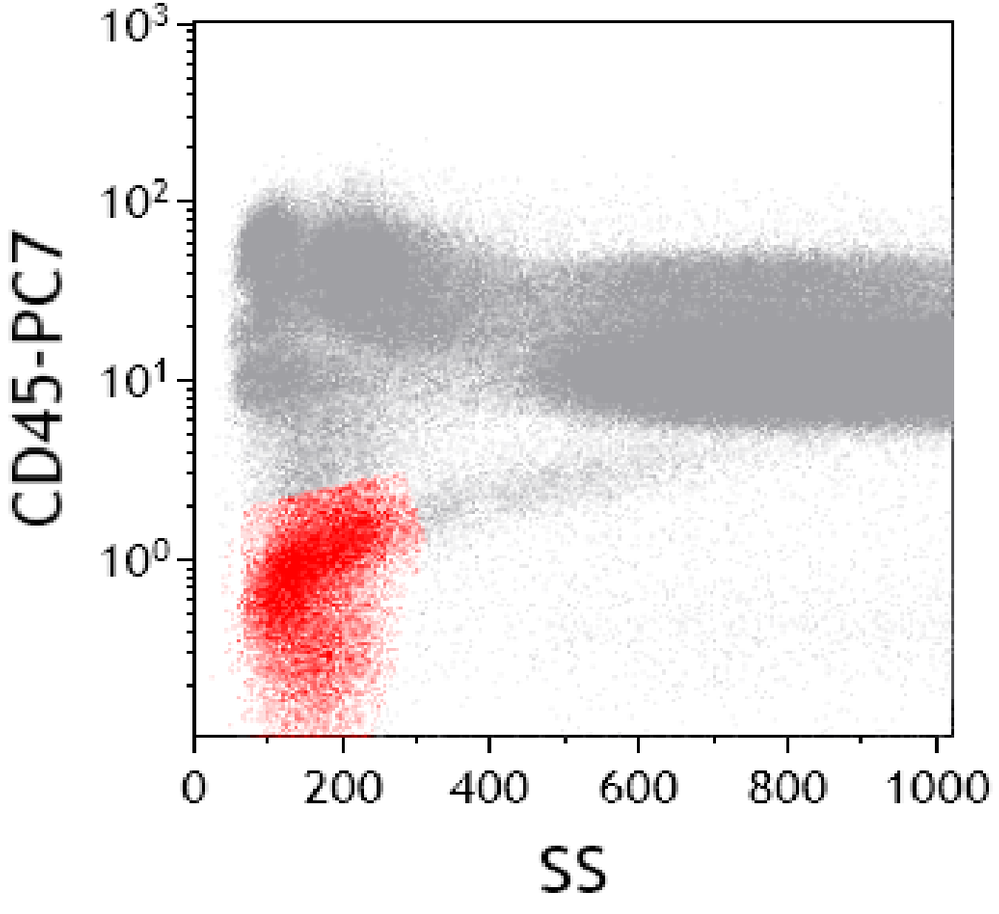
✓ Biopsias (ganglio, bazo, otros tumores)

Médula ósea – CD45 vs SSC



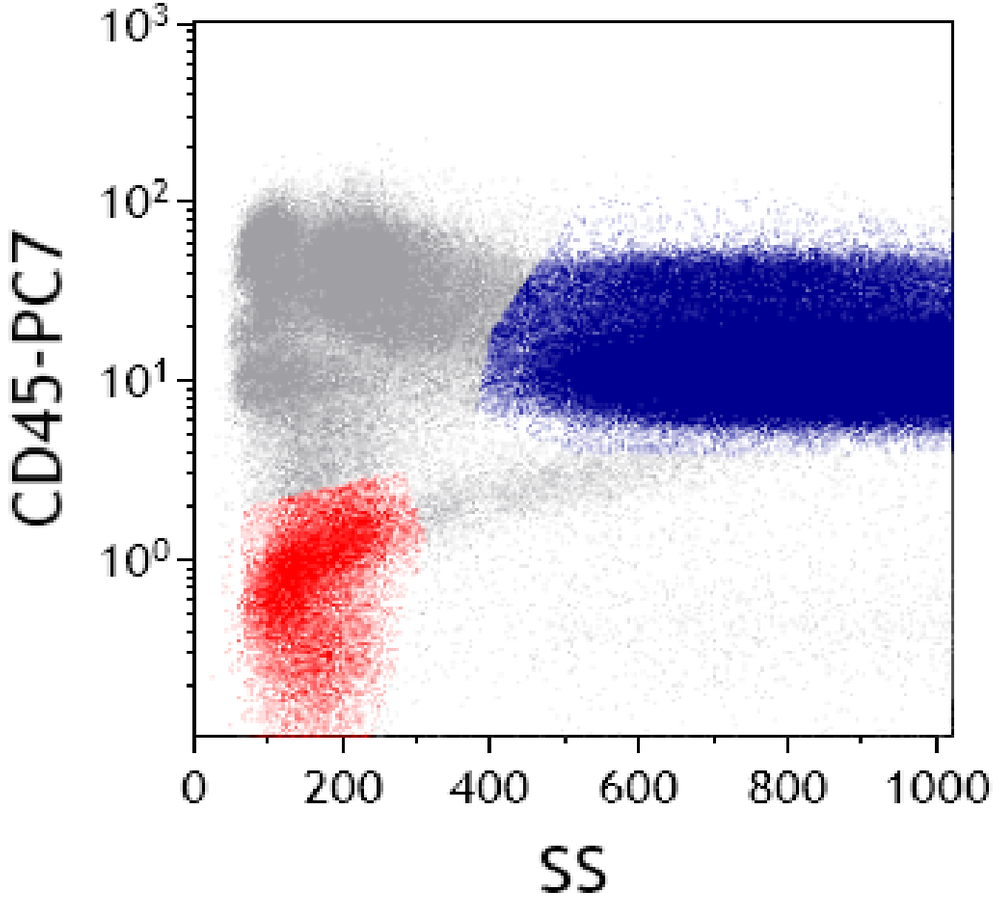
CD45 (ALC)
Antígeno **L**eucocitario **C**omún

Médula ósea – CD45 vs SSC



Serie roja

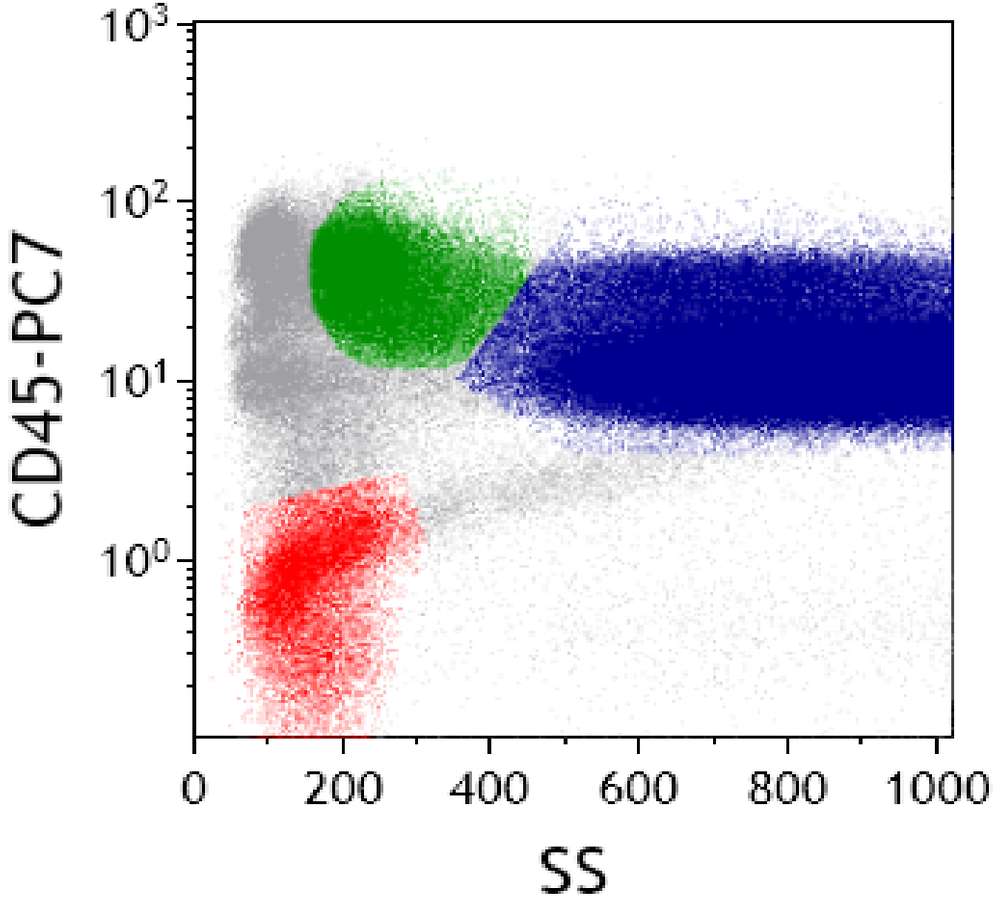
Médula ósea – CD45 vs SSC



Serie roja

Granulocitos

Médula ósea – CD45 vs SSC

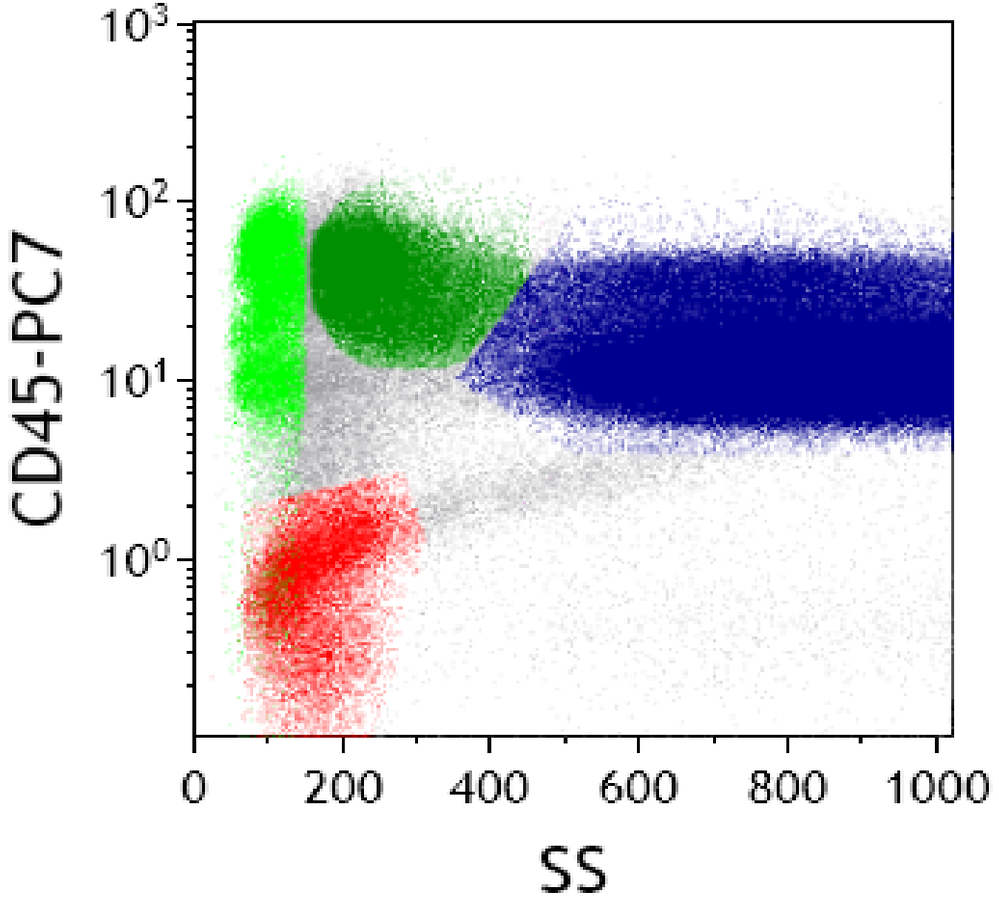


Serie roja

Granulocitos

Monocitos

Médula ósea – CD45 vs SSC



Serie roja

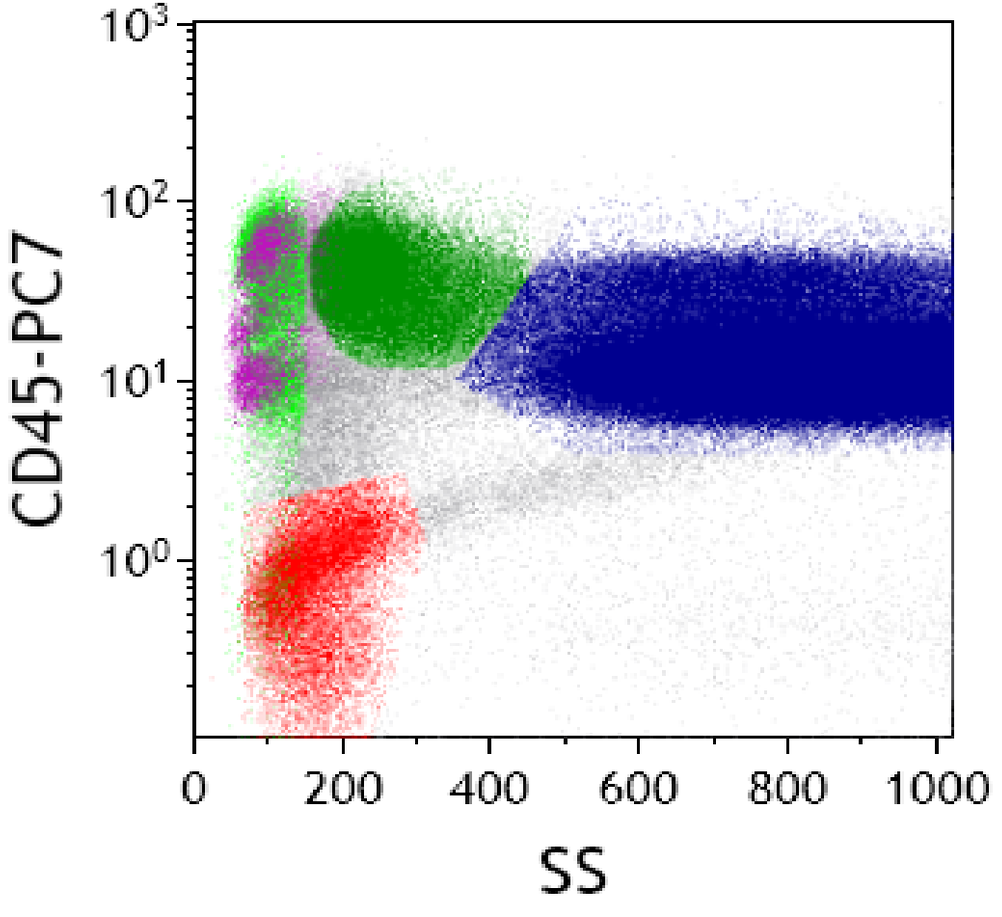
Granulocitos

Monocitos

Linfocitos

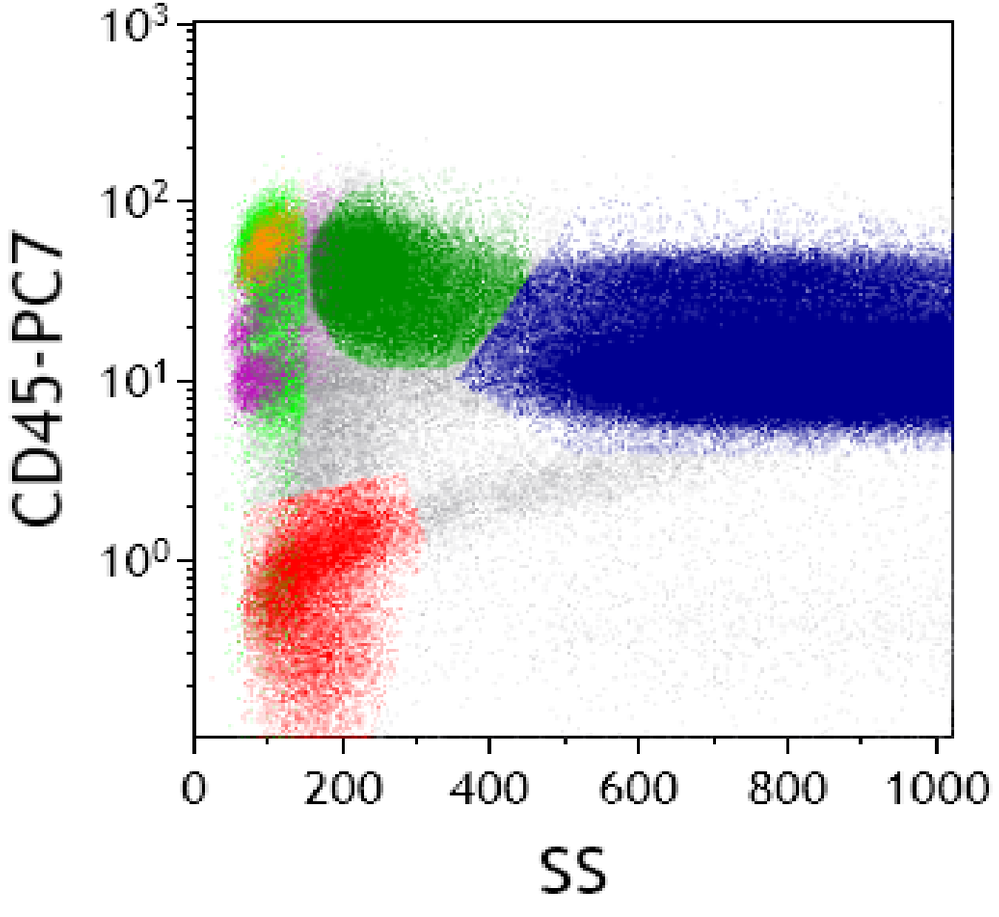
Vall d'Hebron

Médula ósea – CD45 vs SSC



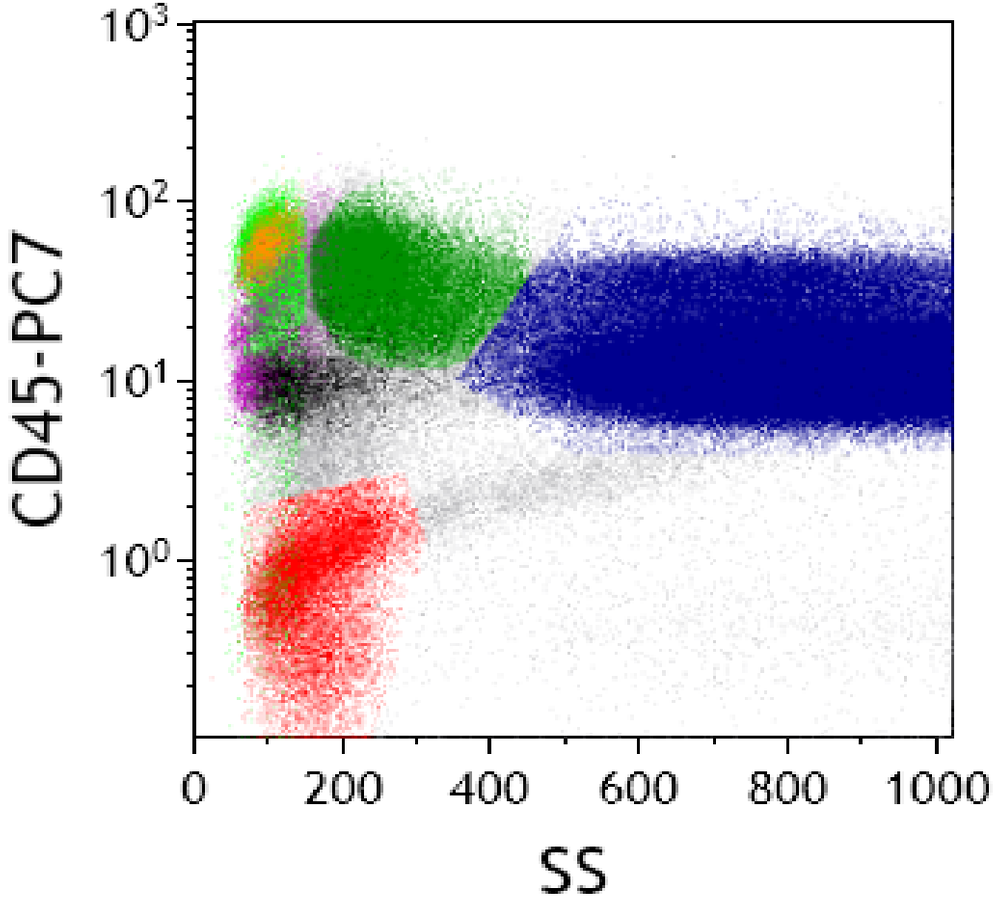
- Serie roja
- Granulocitos
- Monocitos
- Linfocitos T y NK
- Linfocitos B

Médula ósea – CD45 vs SSC



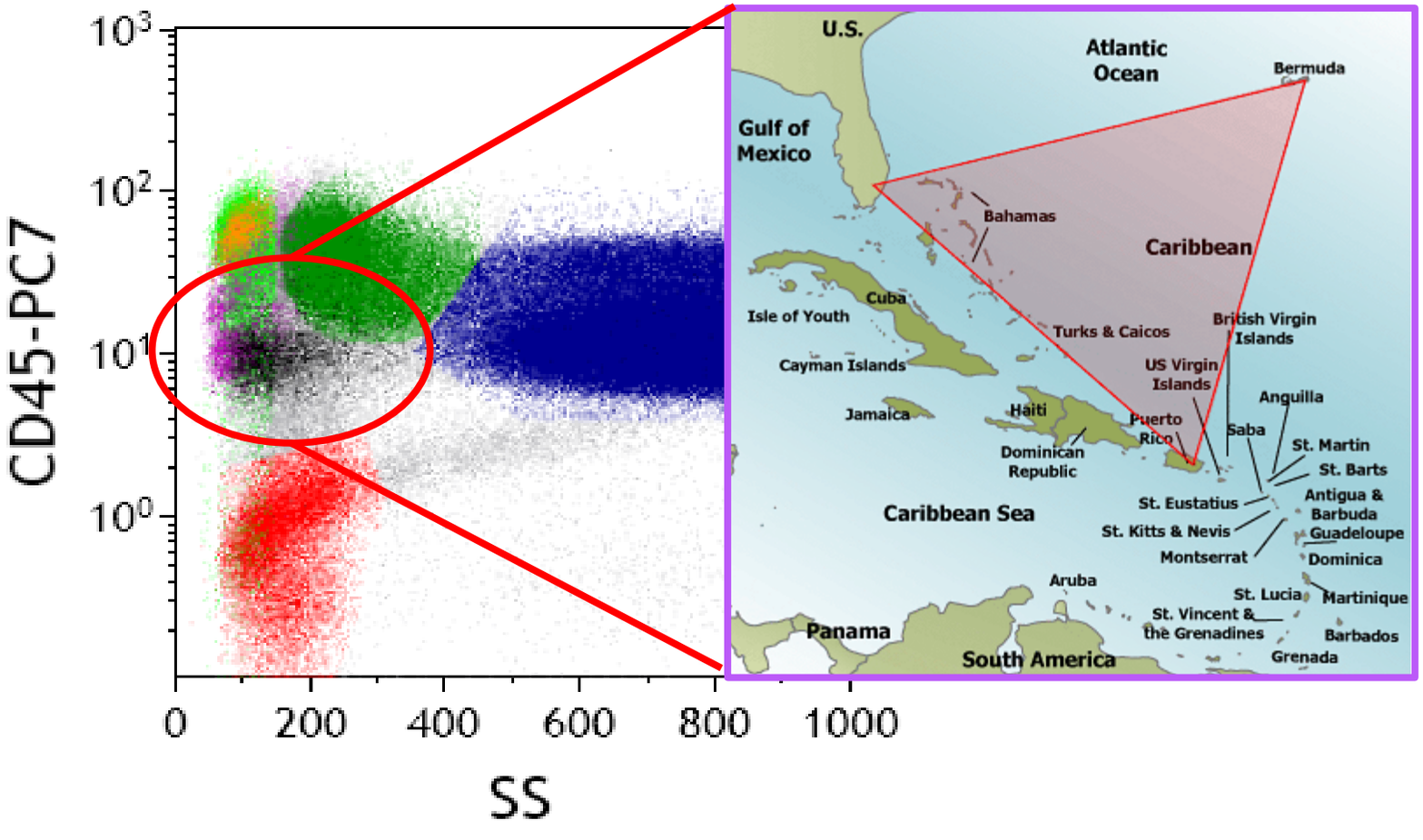
- Serie roja
- Granulocitos
- Monocitos
- Linfocitos T y NK
- Precursores B
- Linfocitos B maduros

Médula ósea – CD45 vs SSC



- Serie roja
- Granulocitos
- Monocitos
- Linfocitos T y NK
- Precursores B
- Linfocitos B maduros
- Precursores mieloides

Médula ósea – CD45 vs SSC

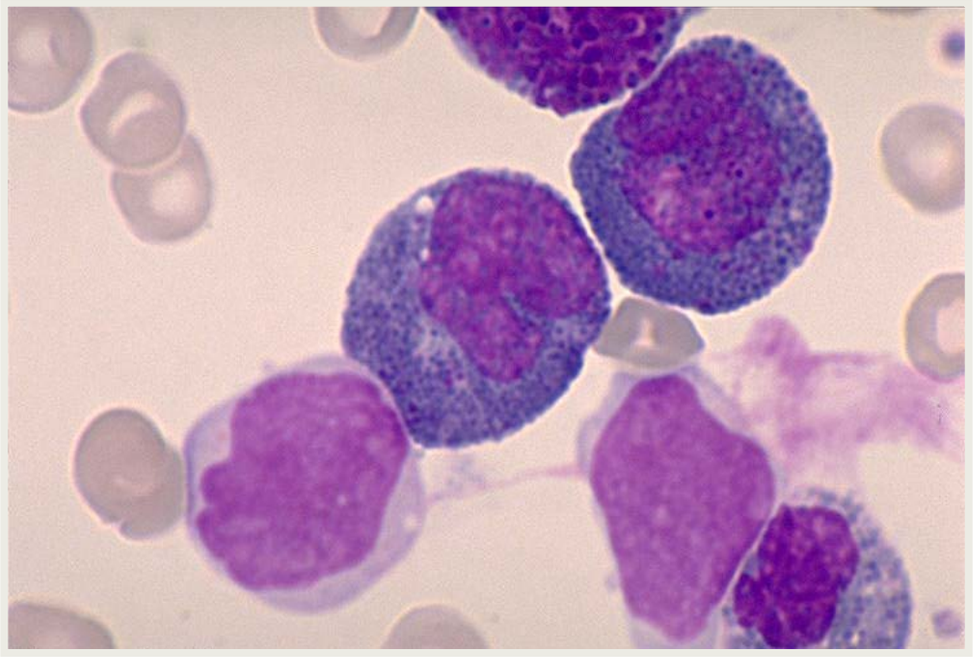


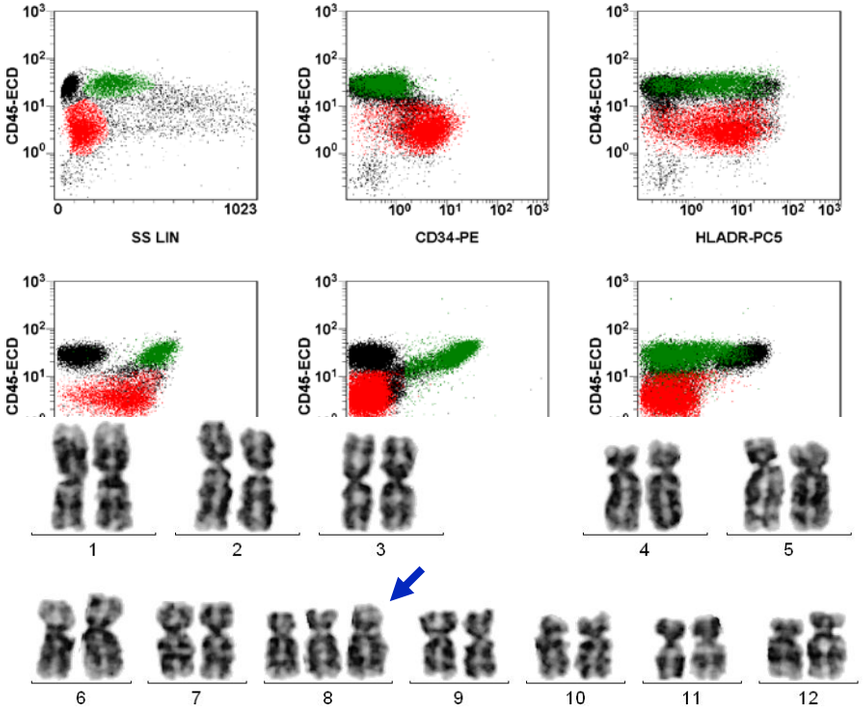
Vall d'Hebron

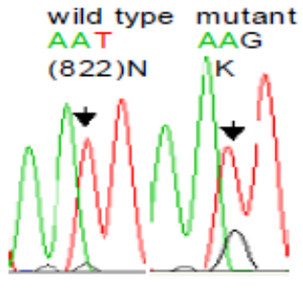
Clasificación de las leucemias agudas (OMS 2016)

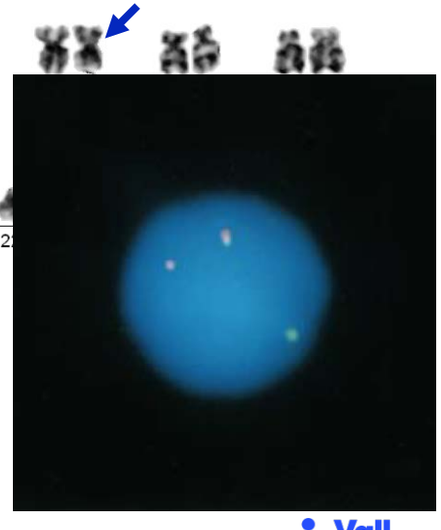
Número de categorías	
Mieloides:	23
Linfoides:	9
Ambiguas:	5
Provisionales:	6
Total:	**43**

Leucemias agudas – Diagnóstico









- ✓ LA Mielomonocítica con eosinofilia
- ✓ CD34+, HLADR+, CD33+,CD14+, CD2+/-
- ✓ 48,XX,inv(16)(p13;q22),+8,+22
- ✓ FISH (LSI CBFB dual color break apart): 1R 1V 1A
- ✓ C-KIT mutado

Vall d'Hebron

Diseño de los paneles de estudio

Debe basarse en

- ✓ Experiencia propia del centro
- ✓ Opinión de expertos:
  - Bethesda 2006
  - EuroFlow 2005 (mínimos)
  - EuroFlow 2009 (paneles 8 FL)
- ✓ Criterios diagnósticos (p.ej. OMS 2016)

Debe tener en cuenta

- ✓ Características del centro

  (Laboratorio centralizado, Hospital comarcal, Hospital 3er nivel, Pediátrico …)

Diagnóstico de leucemia aguda
Panel primario de AcMo

Objetivo: Establecer la línea celular neoplásica

Tub	FITC	PE	ECD	PC7	APC	AAF700	AAF750	PaB	KrO
SLA-cit	cit-MPO	nuc-TdT	cit-CD22	CD7	cit-CD79a	CD34	cit-CD3	m-CD3	CD45

FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-cyanin 7; APC, Allophycocyanin; AAF700, APC-Alexa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

- ✓ **MPO+:** LMA
- ✓ **CD3+ :** LLA-T
- ✓ **CD79a+, CD22+:** LLA-B
- ✓ **MPO- (CD3-, CD79a-):** LMA, M5, M6, M7, otras

Diagnóstico de leucemia aguda
Panel secundario de AcMo

Objetivo: caracterizar la célula neoplásica

Leucemia mieloblástica aguda

Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
LMA1	CD11b	CD15	CD16	CD117	CD33	CD13	CD34	CD10	HLA-DR	CD45
LMA2	CD35	CD300e	CD64	CD117	CD14	CD7	CD34	CD56	HLA-DR	CD45
LMA3	CD71	CD105	CD38	CD117	CD33	CD36	CD34	CD235a	HLA-DR	CD45
LMA4	CD61+41	NG2	CD19	CD117	CD25	CD2	CD34	CD9	HLA-DR	CD45
LMA5	CD141	CD203c	CD4	CD117	CD11c	CD123	CD34	CD56	HLA-DR	CD45

FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-cyanin 7; APC, Allophycocyanin; AAF700, APC-Alexa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

Diagnóstico de leucemia aguda
Panel secundario de AcMo

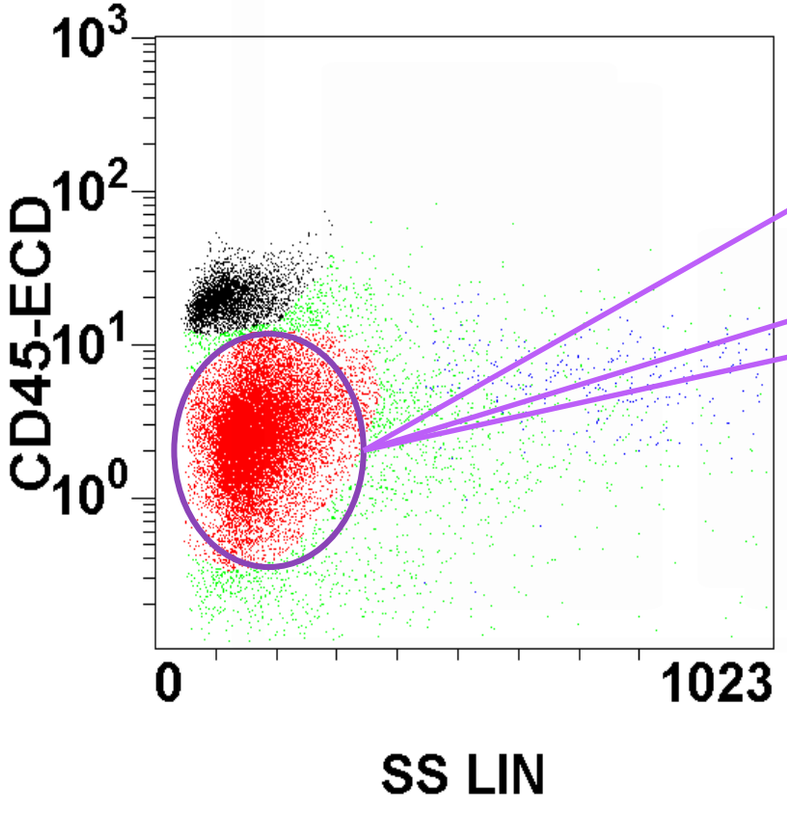
Leucemia mieloblástica aguda

Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
LMA1	CD11b	CD15	CD16	CD117	CD33	CD13	CD34	CD10	HLA-DR	CD45
LMA2	CD35	CD300e	CD64	CD117	CD14	CD7	CD34	CD56	HLA-DR	CD45
LMA3	CD71	CD105	CD38	CD117	CD33	CD36	CD34	CD235a	HLA-DR	CD45
LMA4	CD61+41	NG2	CD19	CD117	CD25	CD2	CD34	CD9	HLA-DR	CD45
LMA5	CD141	CD203c	CD4	CD117	CD11c	CD123	CD34	CD56	HLA-DR	CD45

FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-cyanin 7; APC, Allophycocyanin; AAF700, APC-Alexa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

Diagnóstico de leucemia aguda
Panel secundario de AcMo

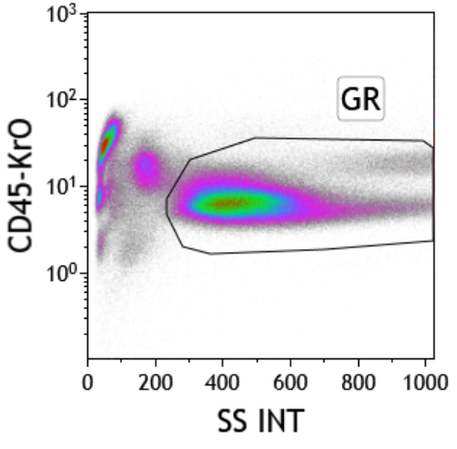
Leucemia mieloblástica aguda

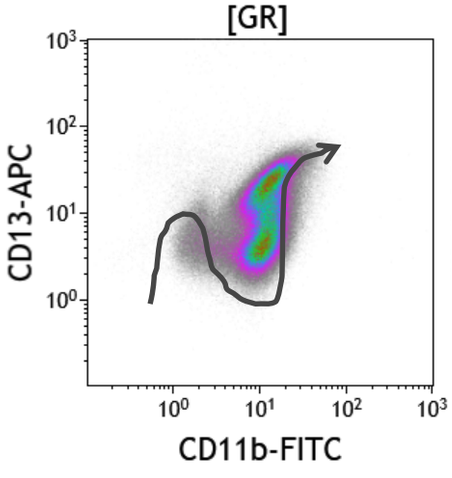


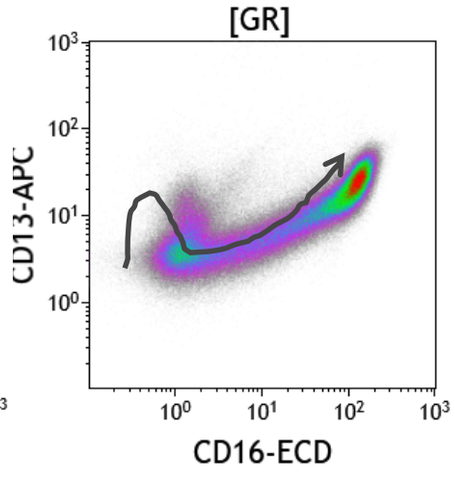
Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
				CD117	CD33	CD13	CD34	CD10	HLA-DR	CD45
				CD117	CD14	CD7	CD34	CD56	HLA-DR	CD45
				CD117	CD33	CD36	CD34	CD235a	HLA-DR	CD45
				CD117	CD25	CD2	CD34	CD9	HLA-DR	CD45
				CD117	CD11c	CD123	CD34	CD56	HLA-DR	CD45

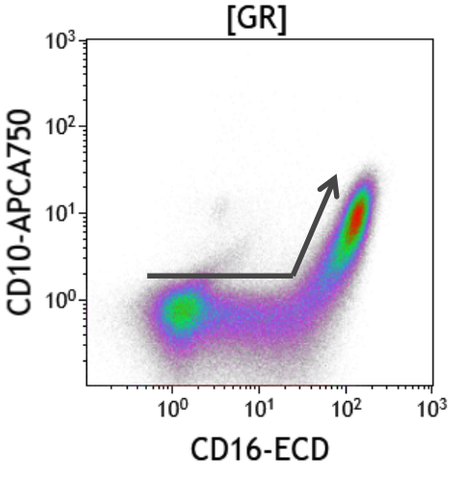
rin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-
exa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

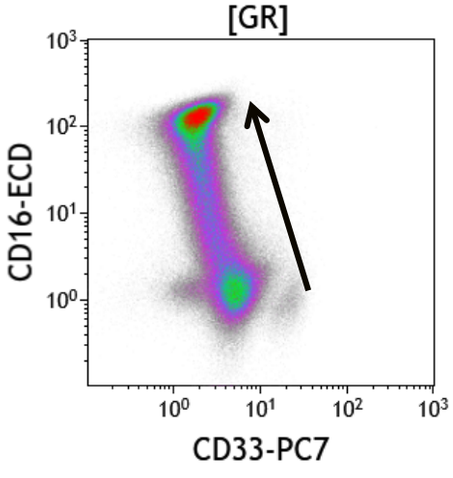
Panel secundario de LMA

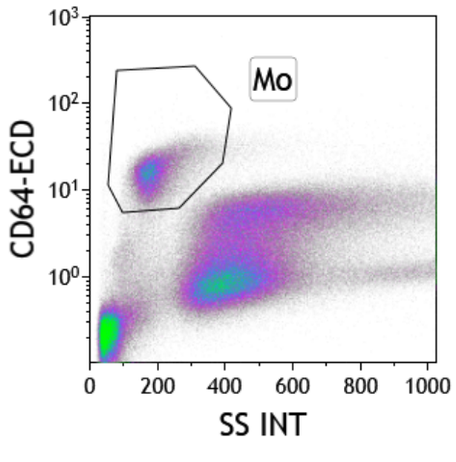


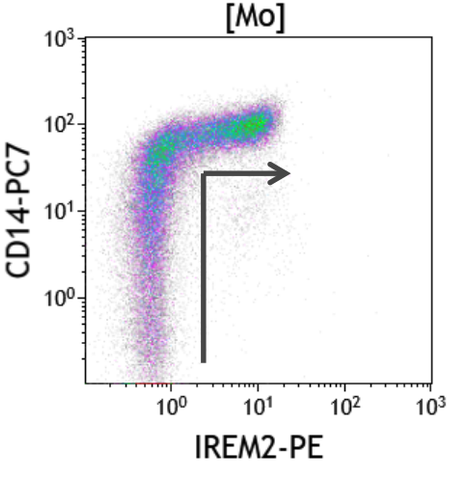


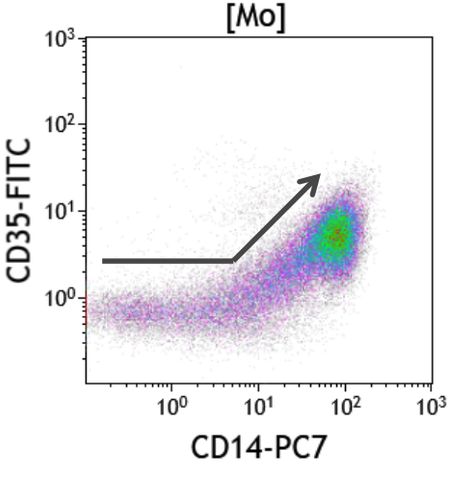












Diagnóstico de leucemia aguda
Panel secundario de AcMo

Leucemia linfoblástica aguda B

Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
LLAB1	CD58	NG2	CD19	CD33	CD10	CD22	CD34	CD38	CD20	CD45
LLAB2	KOR-SA	CD13	CD19	CD24	CD10	CD123	CD34	CD9	CD15	CD45
LLAB3	CD81	CD73	CD19		CD10	CD304	CD34	CD25	CD21	CD45
LLAB-KL	Kappa	Lambda	CD19						CD20	CD45
LLAB-DNA			IP		CD34			CD19		CD45

FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-cyanin 7; APC, Allophycocyanin; AAF700, APC-Alexa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

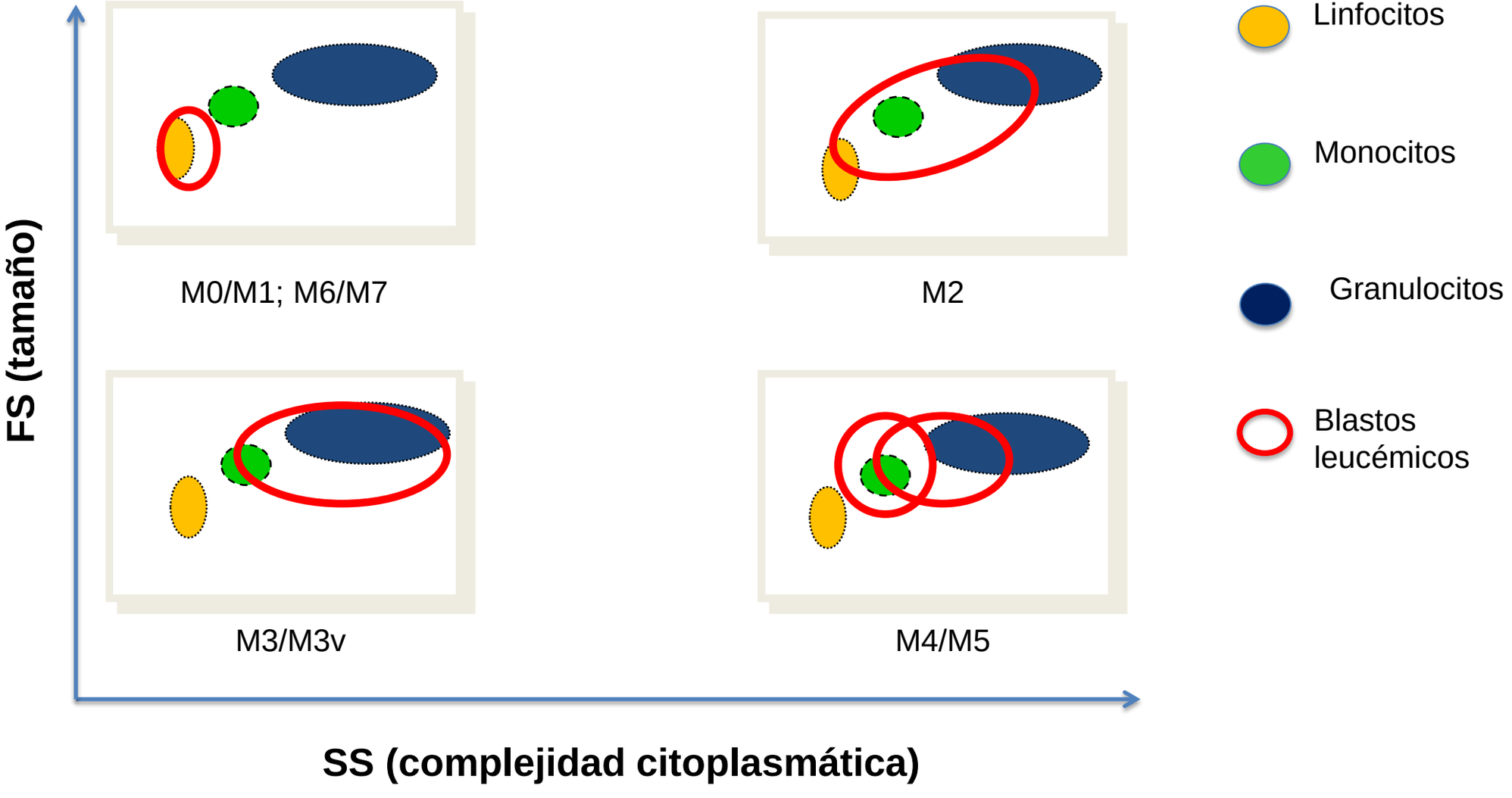
Diagnóstico de leucemia aguda
Panel secundario de AcMo

Leucemia linfoblástica aguda T

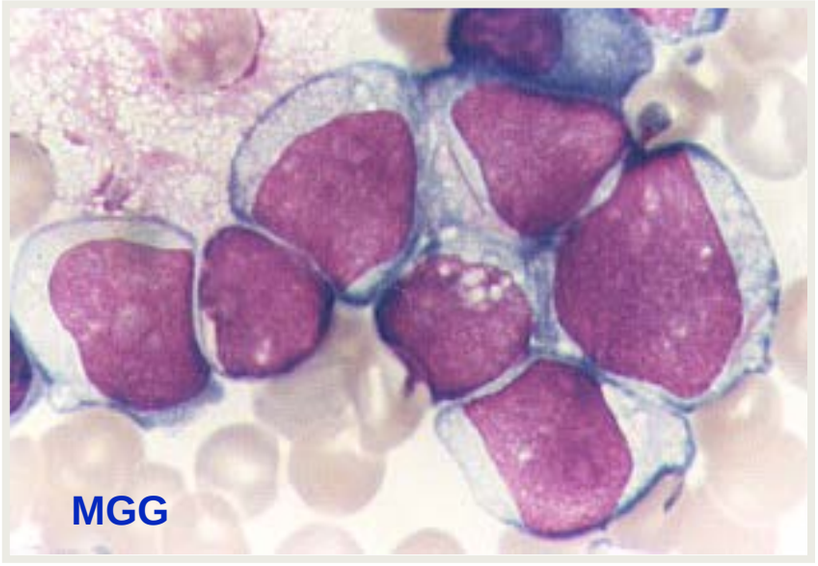
Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
LLAT1	CD2	CD1a	CD3	CD56	CD7	CD5	CD8	CD4	HLA-DR	CD45
LLAT2	TCRgd	CD99	CD3	CD10	CD7	CD13	CD34	CD44	HLA-DR	CD45
LLAT3	CD65	CD33	CD3	CD117	CD7	CD123	CD34		HLA-DR	CD45

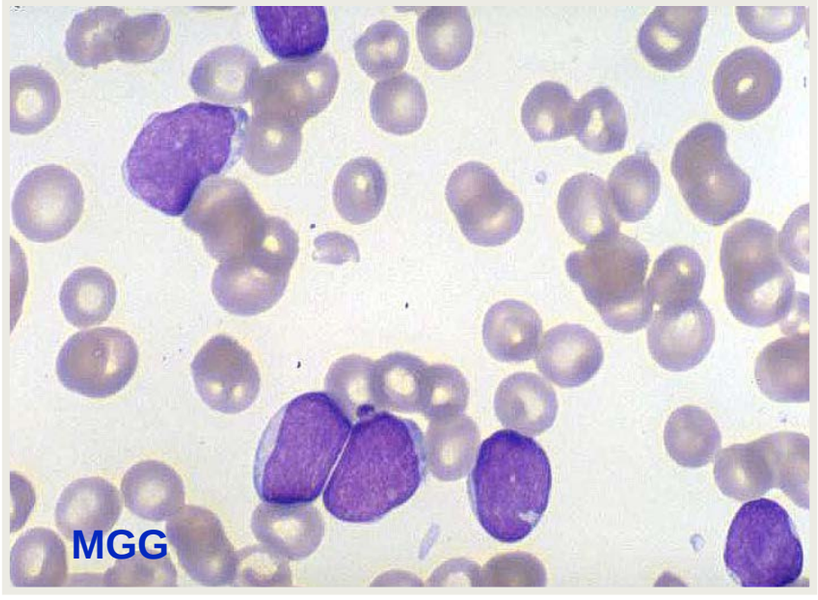
FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-cyanin 7; APC, Allophycocyanin; AAF700, APC-Alexa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

Leucemia aguda – FS vs SS



LMA sin maduración


MGG


MGG

Blastos (tipo I, II) > 90%

MPO > 3% por citoquímica

IF: SS/FS (Ly); CD45 débil

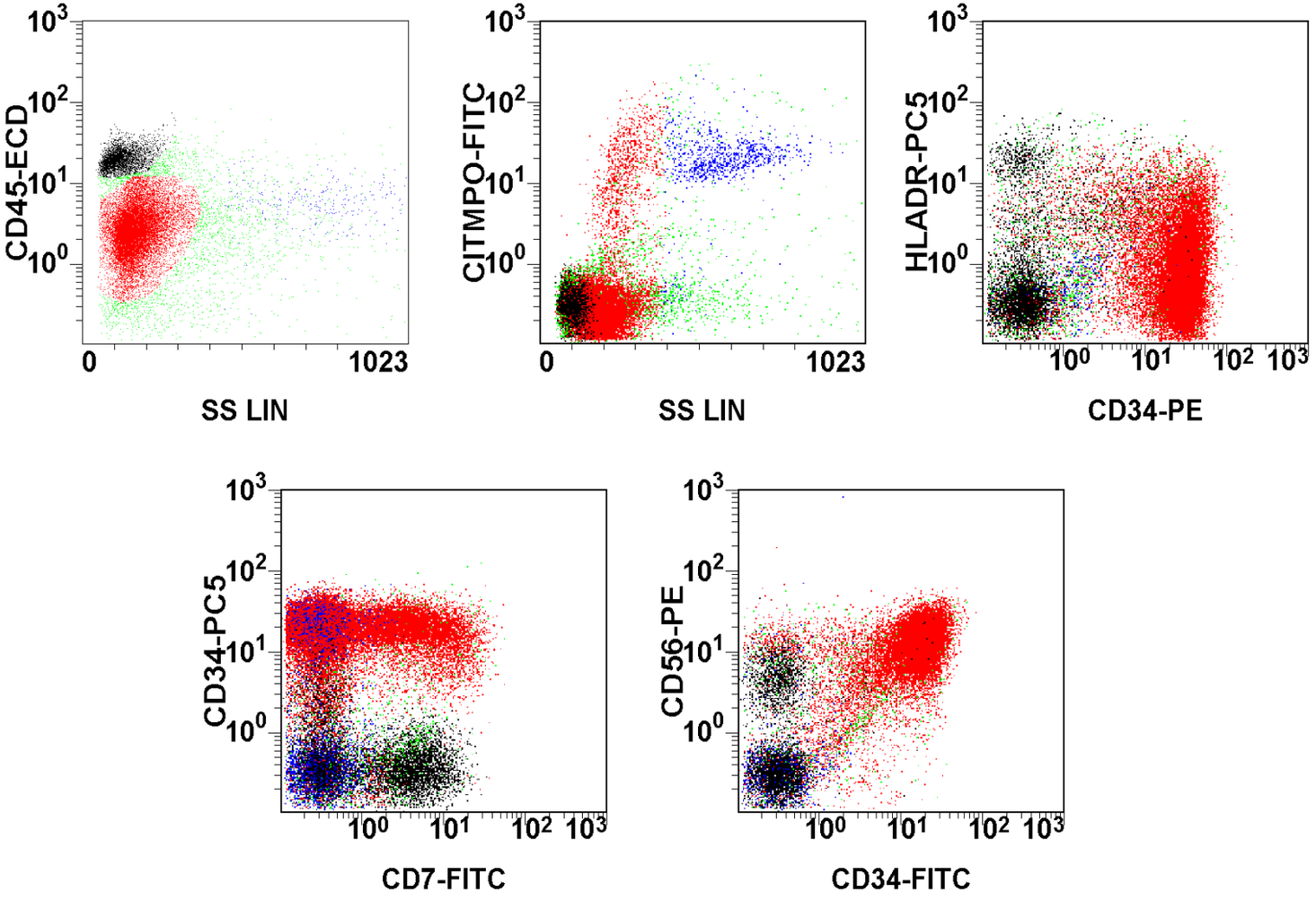
MPO+ y/o CD13+ y/o CD33+ y/o CD65+ y/o c-kit+

CD34+, HLA-DR+

CD15+/-

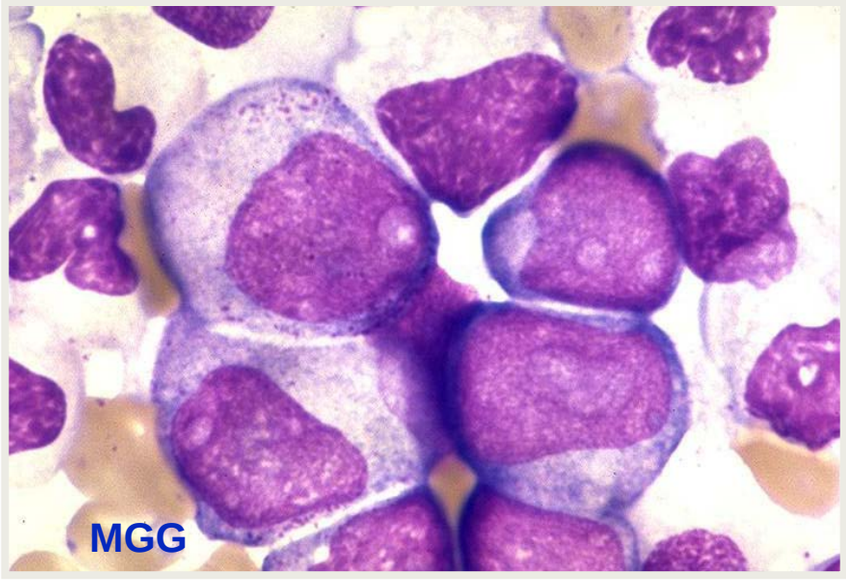
CD19, CD79a, CD3 negativos

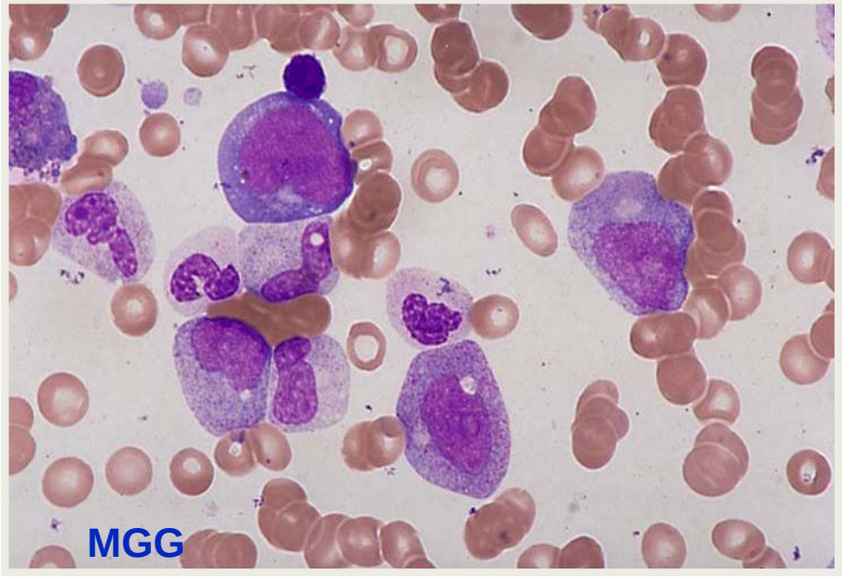
LMA sin maduración



Citogenética: cariotipo complejo

LMA con maduración





Blastos (tipo I, II y III) > 20-89% de CNE

Maduración mieloide > 10%

< 20% componente monocítico

MPO +, abundantes Auer

IF: elevado SS; CD45 débil

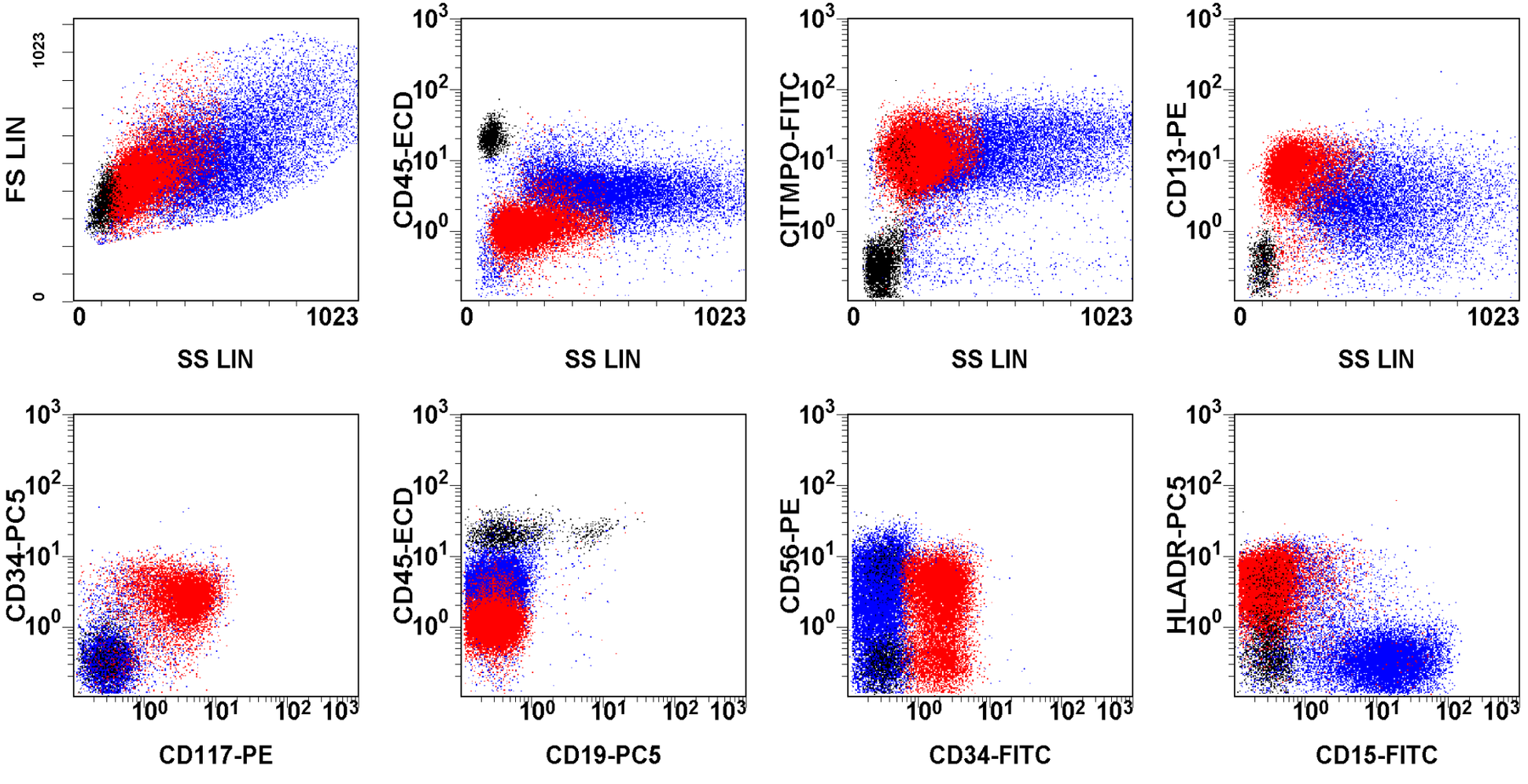
My +; CD13 > CD33

CD34+ (< Fr e intenso que M0/M1), HLA-DR+

CD15+ (> Fr e intenso que M0/M1)

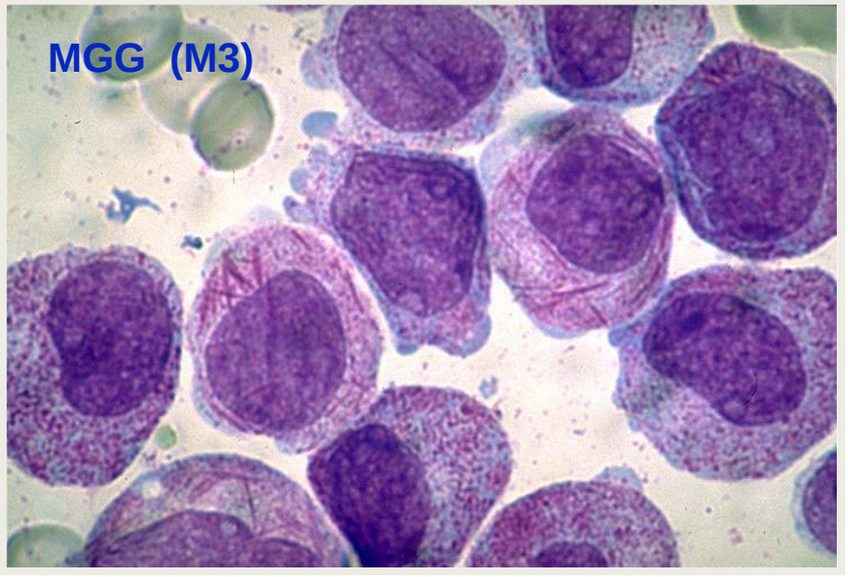
CD19+, CD56+: **t(8;21)**

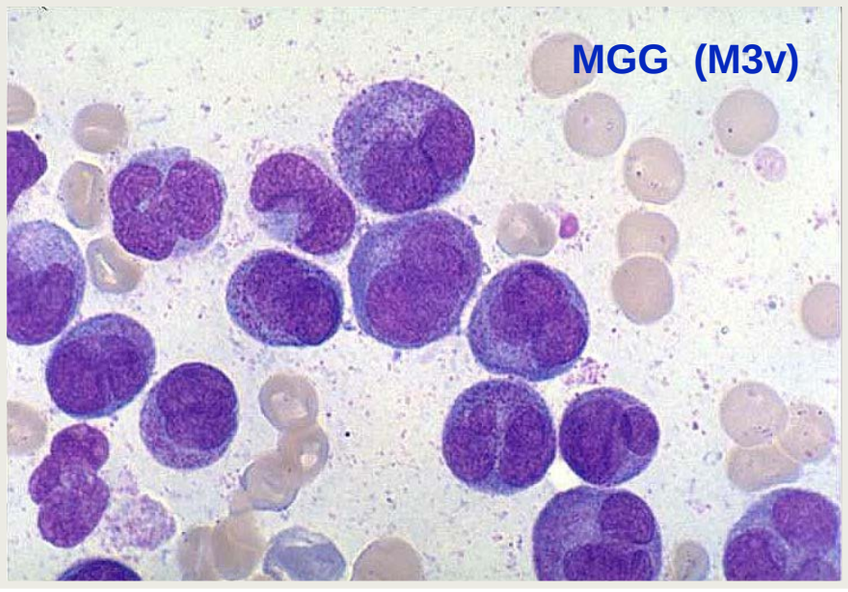
LMA con maduración



Citogenética: cariotipo normal

Leucemia promielocítica aguda





Promielocitos atípicos

MPO intensa; astillas (*faggot cells*)

t(15;17)(q22;q11) PML-RARα

IF: elevado SS; CD45 débil

CD34-; HLA-DR-

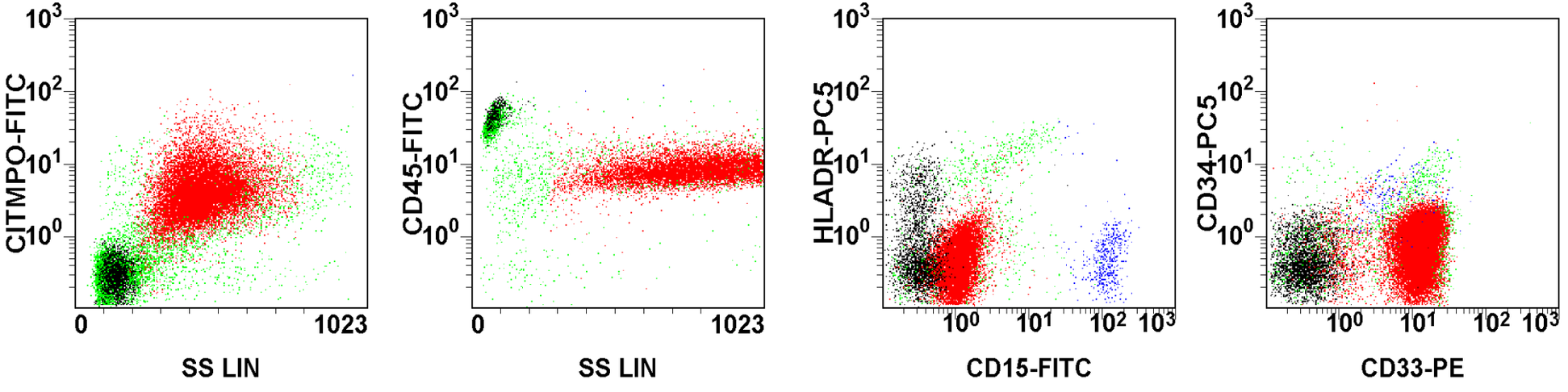
CD13 débil y heterogéneo

Stop madurativo en CD15

M3v: CD2+ **t(15;17)**

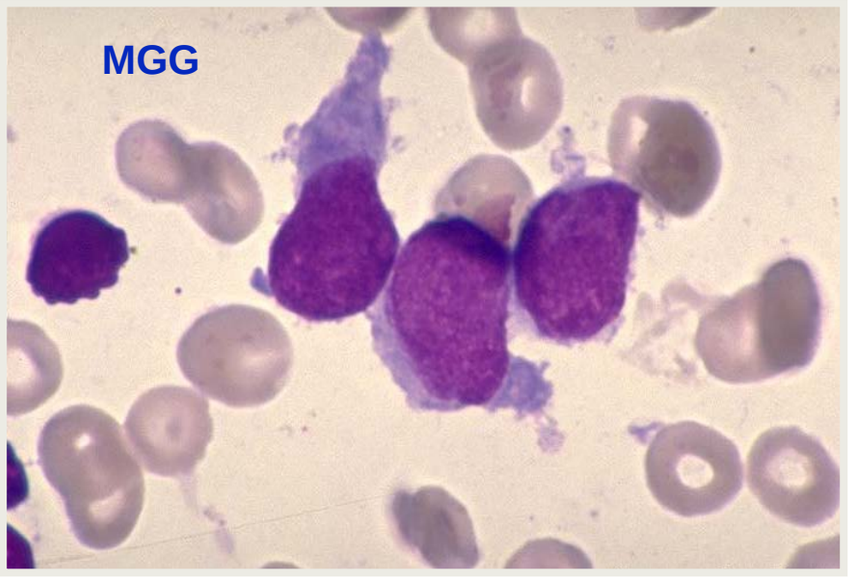
CD56+ **t(11;17)** (PLZF-RAR α)

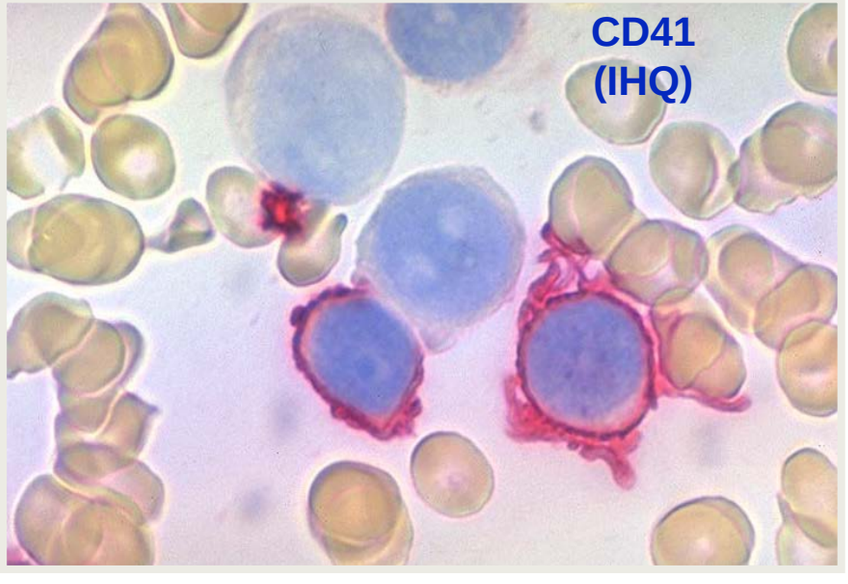
Leucemia promielocítica aguda



Citogenética: 46,XX,t(15;17)(q22;q11)

Leucemia megacarioblástica





Blastos (tipo I y II) > 20% de CNE

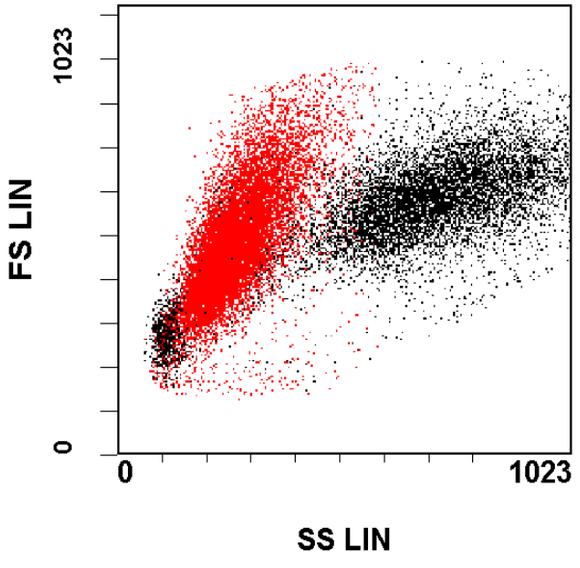
Morfología indiferenciada

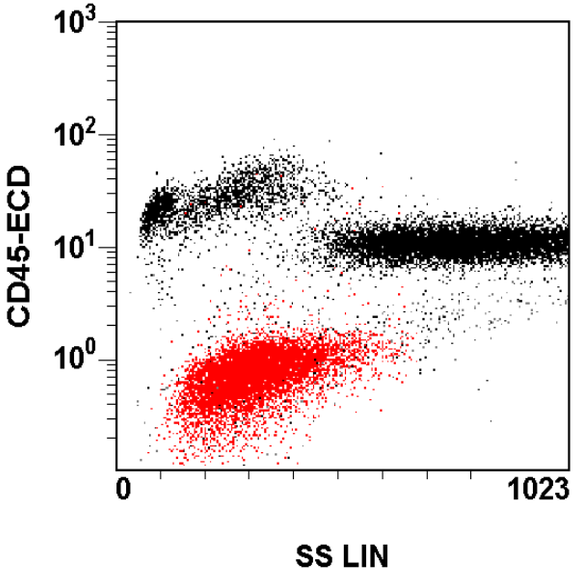
MPO negativa

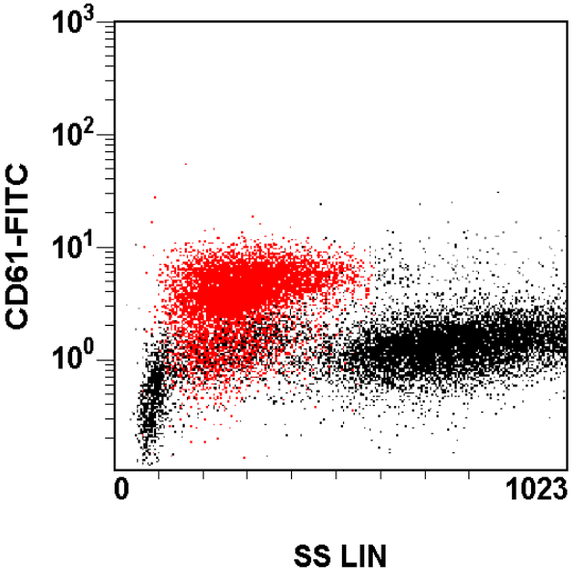
IF: CD34+, CD13+, CD33+

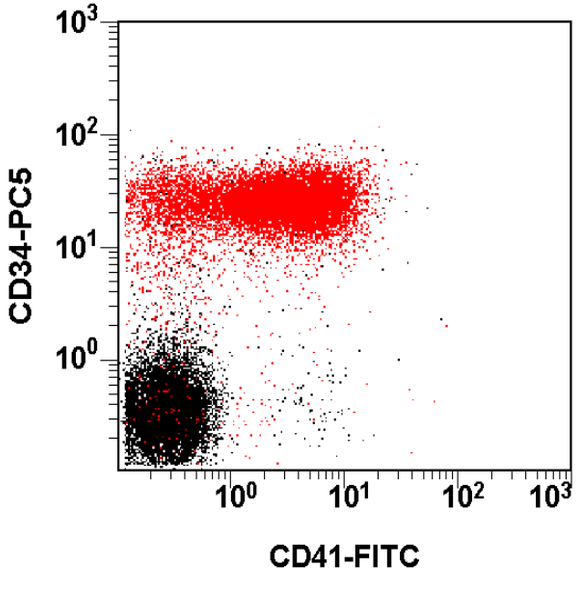
CD61+, CD41+

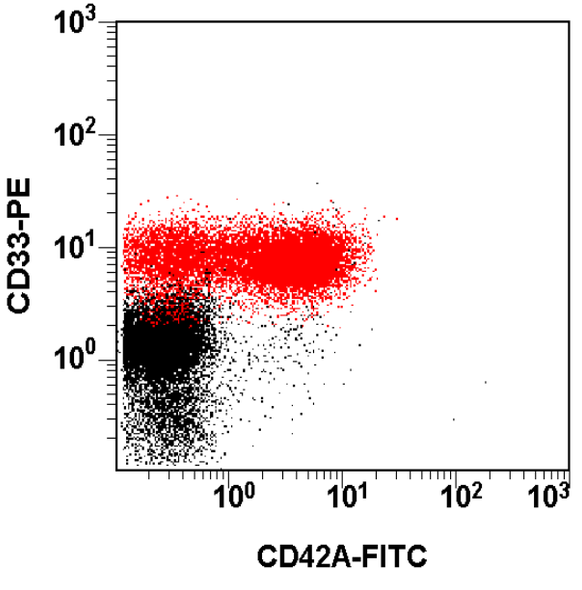
Leucemia megacarioblástica











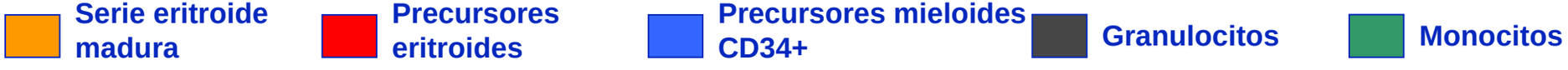
CD45 –

CD61 (GpIIIa) +

CD41 (GpIIb) +

CD42a (GpIX) +

Eritroleucemia



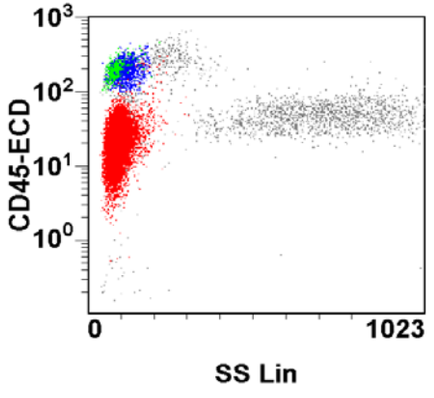
Leucemia aguda de línea ambigua

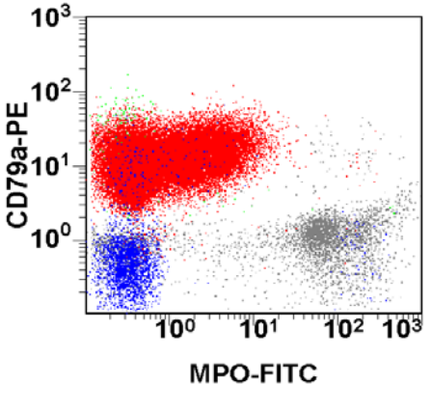
1. **LA Bilineal** ≥ 2 poblaciones diferentes, una de ellas mieloide.

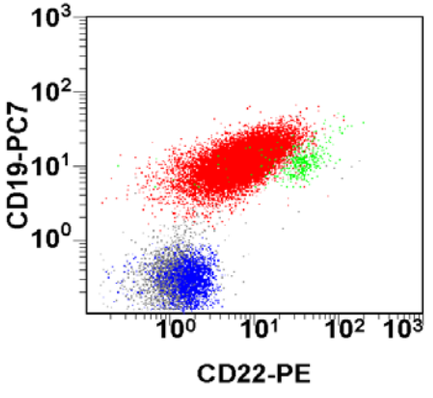
2. **LA Bifenotípica**: 1 sola población

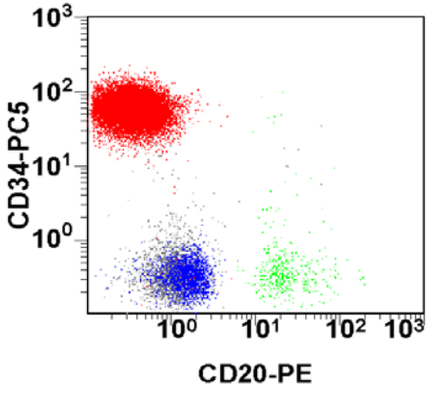
Línea mieloide:	MPO (CF, IHQ o citoquímica)
Línea monocítica:	CD11c, CD14, CD64, lisozima, esterasas inesp.
Línea B:	CD19hi + (≥ 1) CD10, CD22, CD79a o CD19lo + (≥ 2) CD10, CD22, CD79a
Línea T:	CD3cit o CD3sup

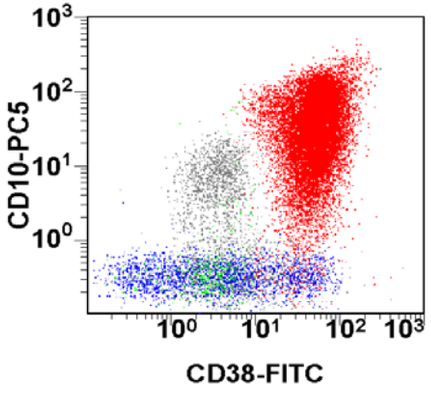
Leucemia aguda de línea ambigua

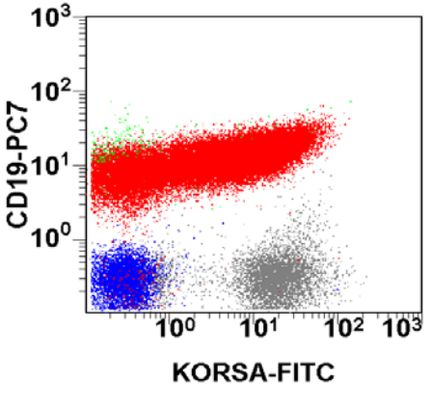


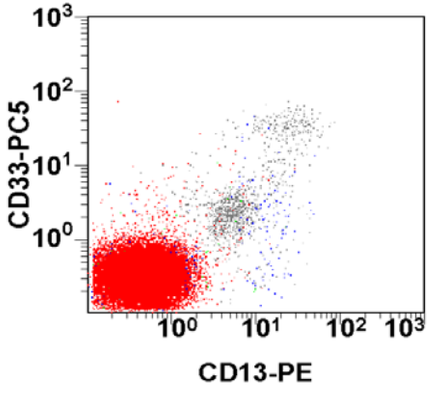


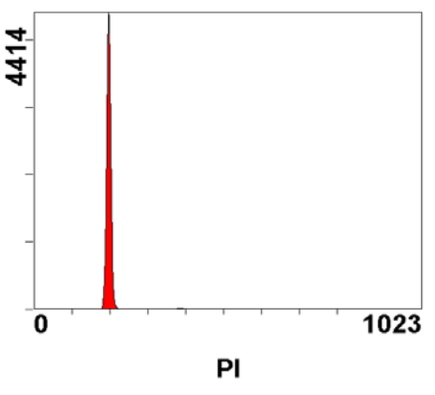












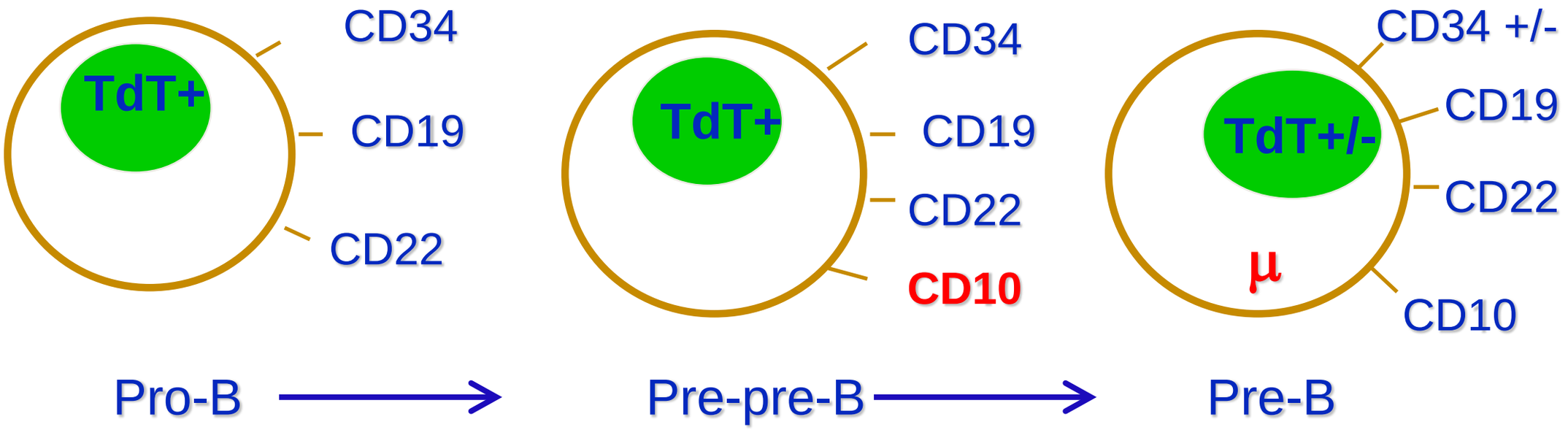
Linfocitos T maduros **Linfocitos B maduros** **Blastos CD19+ MPO+/-**

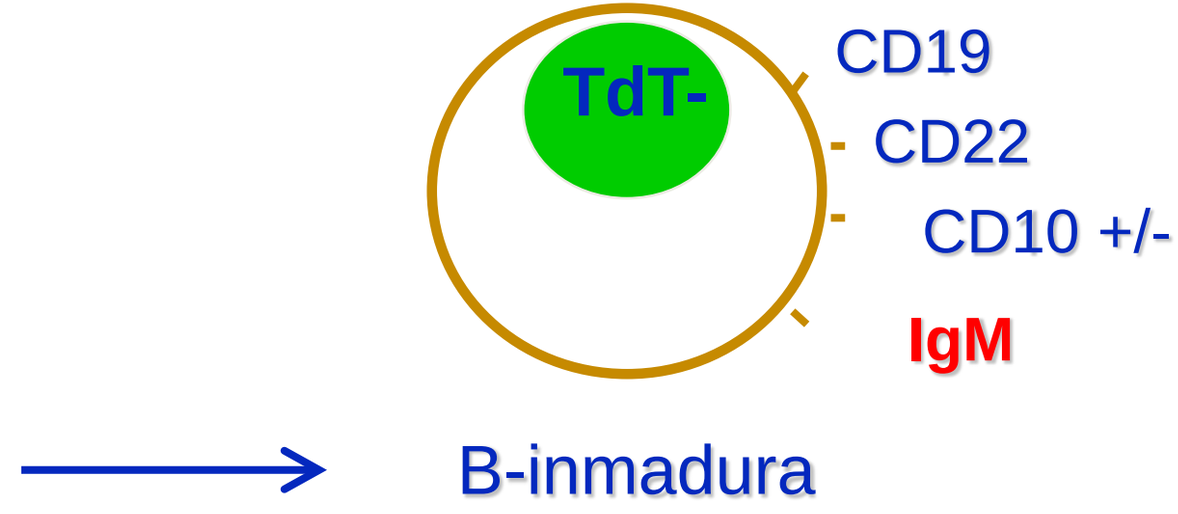
Vall d'Hebron

LMA: patrones “específicos”

Mínimamente diferenciada	HLA-DR+, CD34+, CD117+, CD33+, CD7+/-, CD4+/-
Eritroleucemia	HLA-DR+, CD34+, CD33+/-, CD13+/-, Glicoforina A+
	CD71, CD105 y CD36+ (inespecíficos)
Megacarioblástica	CD41 (GpIIb-IIIa)+, CD61 (GpIIIa)+
Línea ambigua	My/ B; My/T; My/B/T
CBFβ-MYH11	CD34+, CD13+, CD2+ (31%)
PML-RARα	HLA-DR-, CD34-, CD15s+, CD13+ heterogéneo. Stop madurativo. Autofluorescencia. (Sensibilidad 100% ; especificidad 99%)
RUNX1-RUNX1T1	CD19+, CD56+, CD34+++
NPM1A mutada	My+, Mo+ (CD14, CD11b), CD34-
FLT3-ITD	CD123+, CD25+, CD99+

Diferenciación linfoide B





LLA-B (EGIL)

1) **LLA pro-B (BI):** TdT+, CD34+, HLA-DR+, CD38+, CD19+ y/o CD22+ y/o CD79a+, CD10-, Ig(cit)-, Ig(sup)-.

2) **LLA B común (BII):** TdT+, CD34+, HLA-DR+, CD38+, CD19+ y/o CD22+ y/o CD79a+, CD10+, Ig(cit)-, Ig(sup)-.

3) **LLA pre-B (BIII):** TdT+/-, CD34-/+, HLA-DR+, CD38+/-, CD19+, CD22+, CD79a+, CD10+/-, Ig(cit)+, Ig(sup)-.

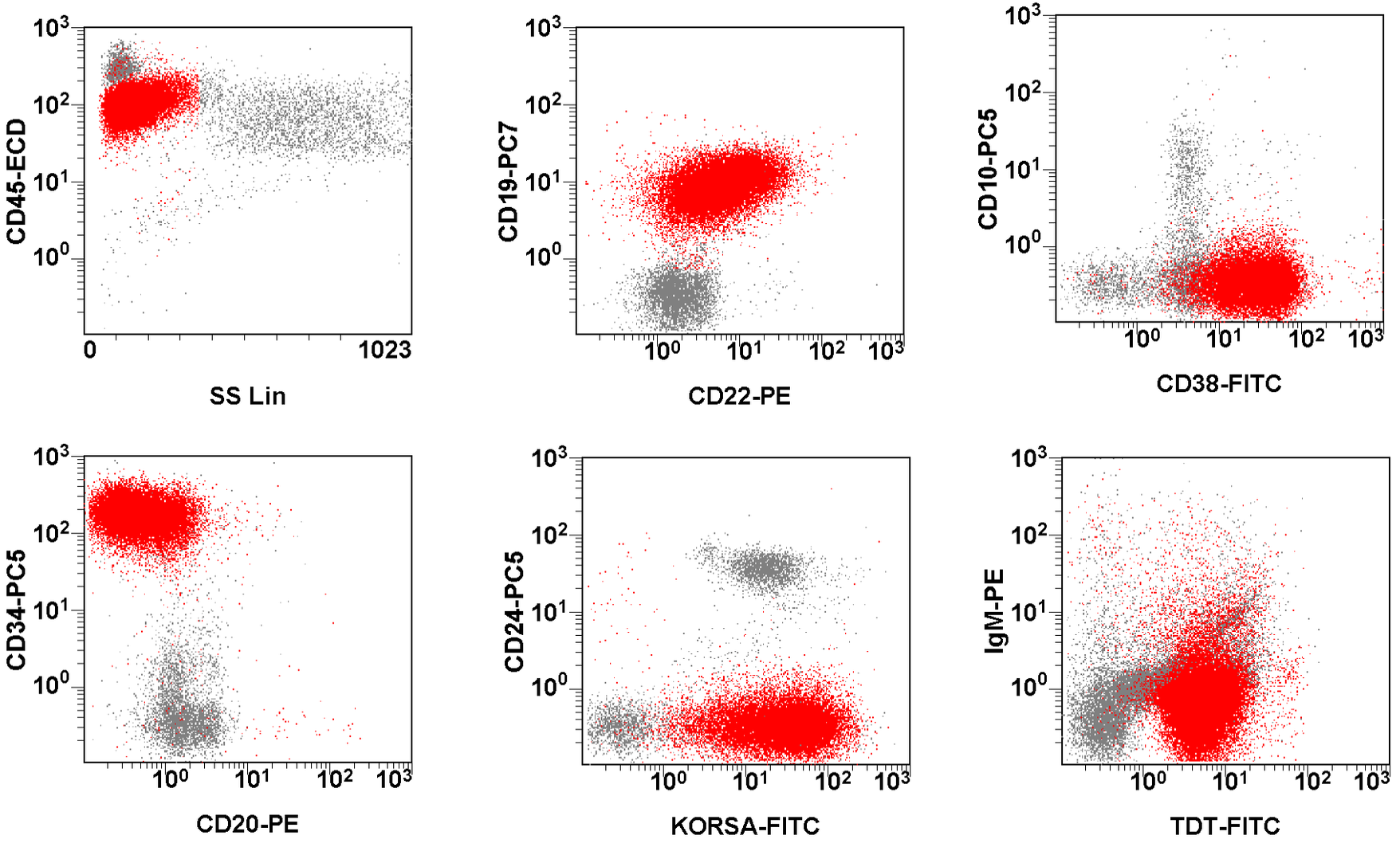
4) **LLA B madura (BIV):** TdT-, CD34-, HLA-DR+, CD38-/+, CD19+, CD22+, CD79a+, CD10+/-, Ig(cit)-, Ig(sup)+

Entre los tipos BIII y BIV se encontraría una LLA-B inmadura “de transición” caracterizada por la pérdida del TdT y la aparición de cadenas pesadas μ de las Ig en superficie sin cadenas ligeras.

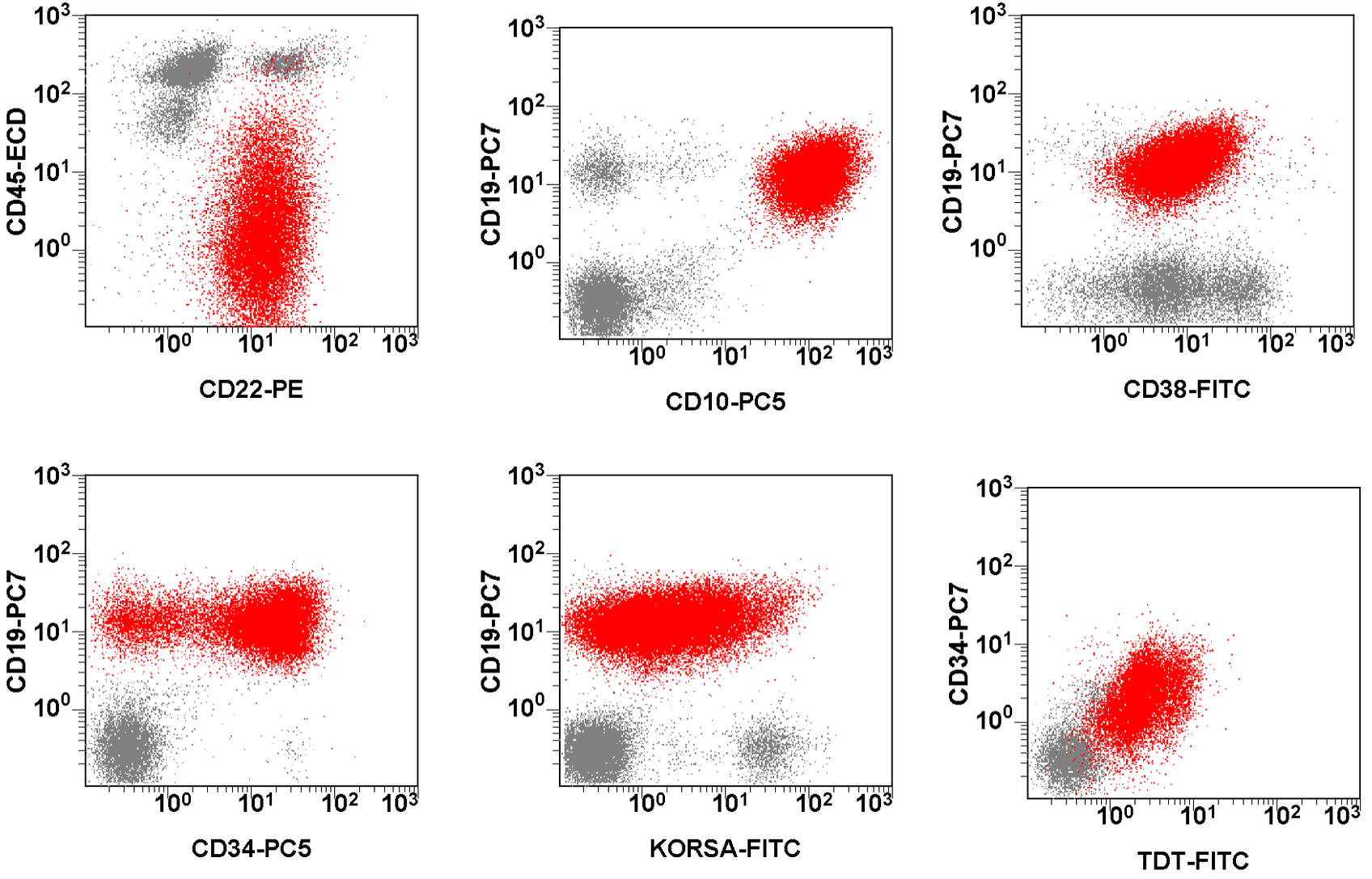
LLA-B: patrones “específicos”

t(9;22); BCR-ABL:	KOR-SA^{+}, CD66^{+}, CD38 $^{het / low}$, CD34 y CD10 ^{hom}, CD13+
11q23 (MLL):	Pro-B, CD9+, CD24-, CD15^{+}, 7.1 (NG2)$^{+}$, My^{+}
t(1;19); E2A-PBX1:	Pre-B, CD9hi, CD34-
t(12;21); ETV6-RUNX1:	CD45$^{-/d}$, CD20^{-}, CD10^{+++}, CD34 bimodal (↑ CV), CD9-
t(5;14); IL3-IGH:	CD19+, CD10+. Bajo % de blastos. Eosinofilia

LLA-B 'pro-B'

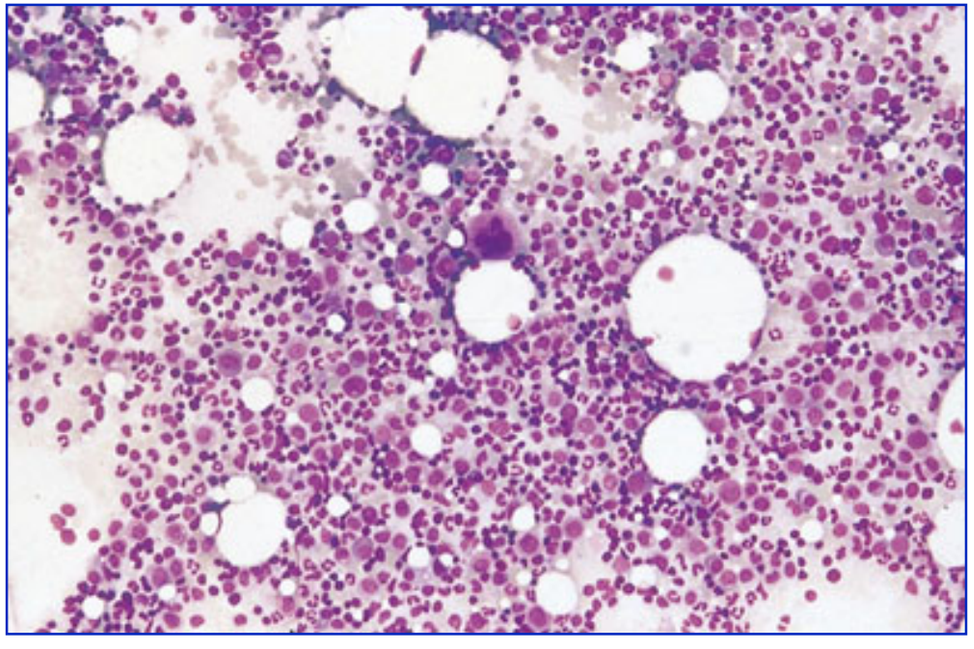

CD45-ECD
SS Lin
0
1023
CD19-PC7
CD22-PE
CD10-PC5
CD38-FITC
CD34-PC5
CD20-PE
CD24-PC5
KORSA-FITC
IgM-PE
TDT-FITC

LLA-B 'B común'


CD45-ECD
CD22-PE
CD19-PC7
CD10-PC5
CD19-PC7
CD38-FITC
CD19-PC7
CD34-PC5
CD19-PC7
KORSA-FITC
CD34-PC7
TDT-FITC

Enfermedad mínima residual

Remisión completa morfológica

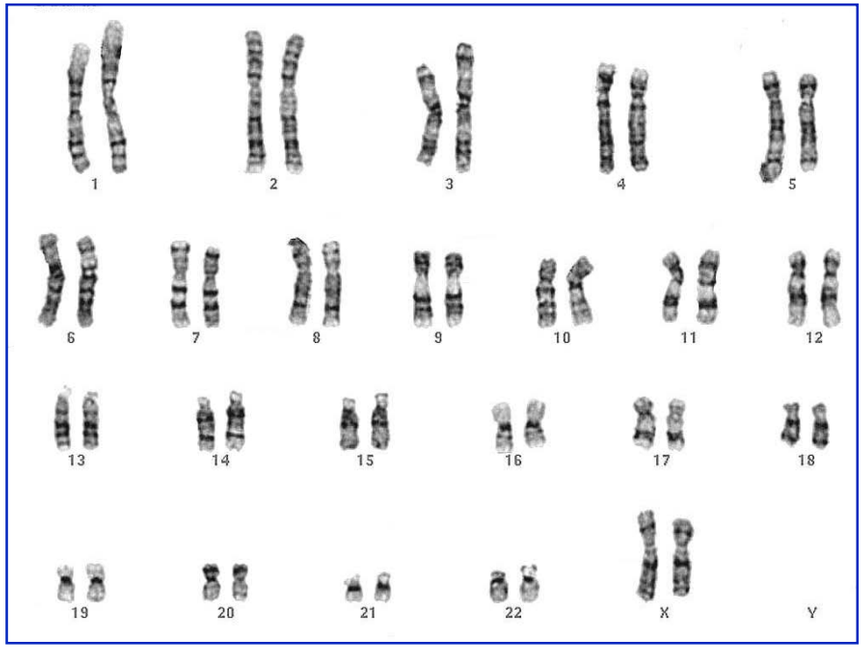


AMO < 5% blastos

SP Plaquetas > $100x10^9$/ L

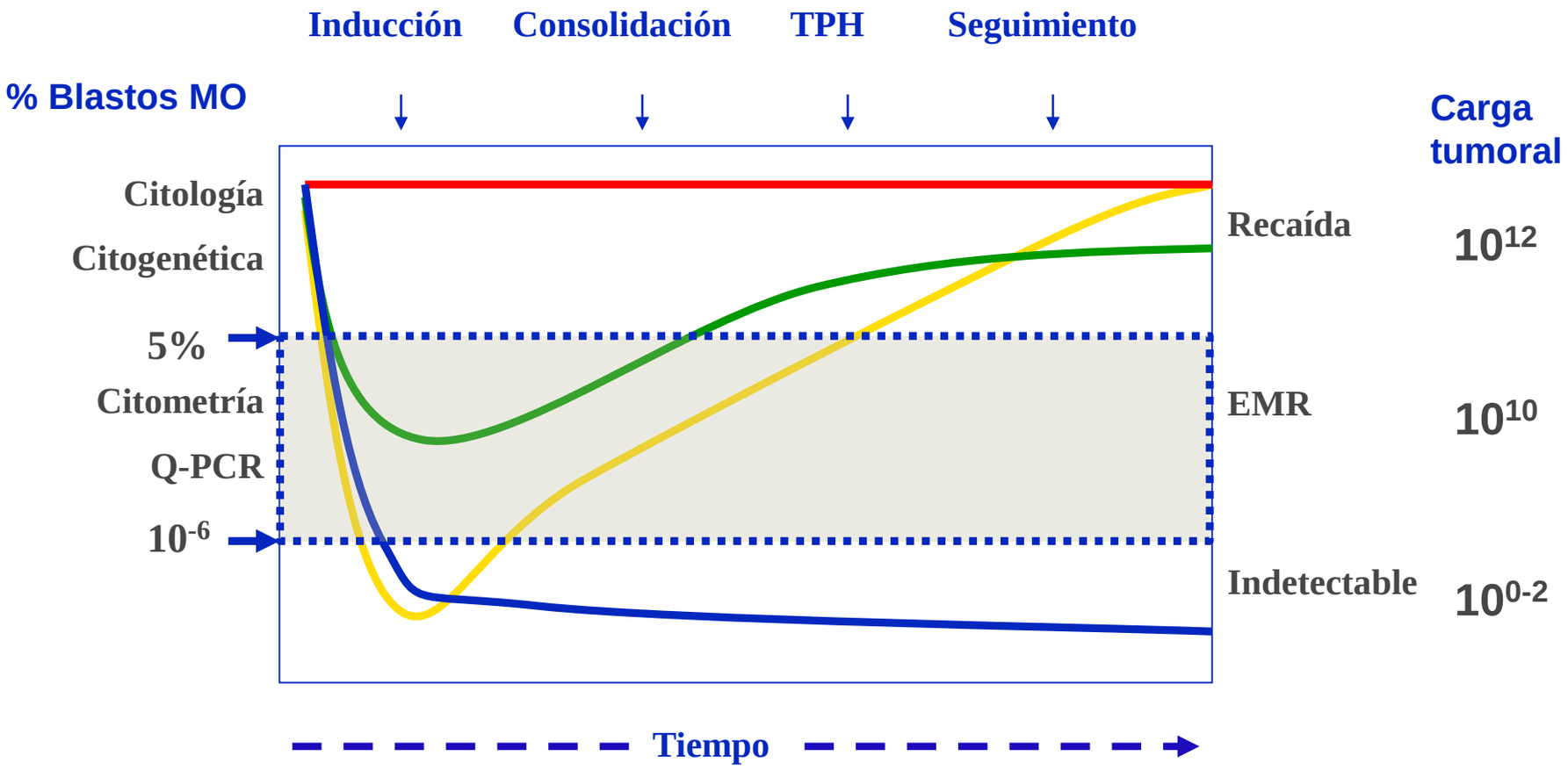
RAN > $1x10^9$/ L

Remisión completa citogenética



Cariotipo normal

Evaluación de la respuesta al tratamiento



EMR - LLA

Coustan-Smith E et al. Blood 2002 *(St. Jude)*:
EMR día +19

CIR

≥ 0.01%: 32.2% a los 5a.

< 0.01%: 6.0% a los 5a.

Vidriales MB et al. Blood 2003 (*Salamanca)*:
EMR día +14

≥0.5% RFS 16 m; <0.5% RFS NR

<0.03% RFS a los 5a de 90%

Basso G et al. JCO 2009 (*AIEOP-BFM-ALL*):
EMR día +15

CIR

< 0.1% (standard): 7.5% als 5a.

0.1-10% (intermedio): 17.5% als 5a.

> 10% (alto): 47.2% als 5a.

Post-inducción

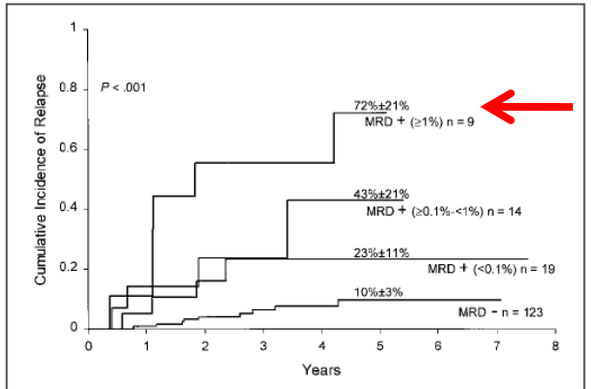
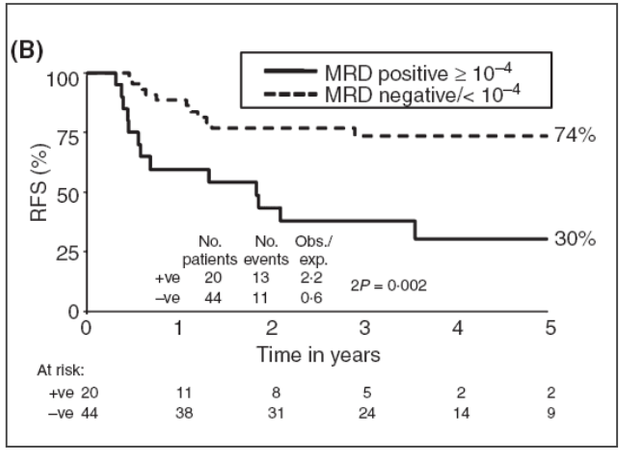


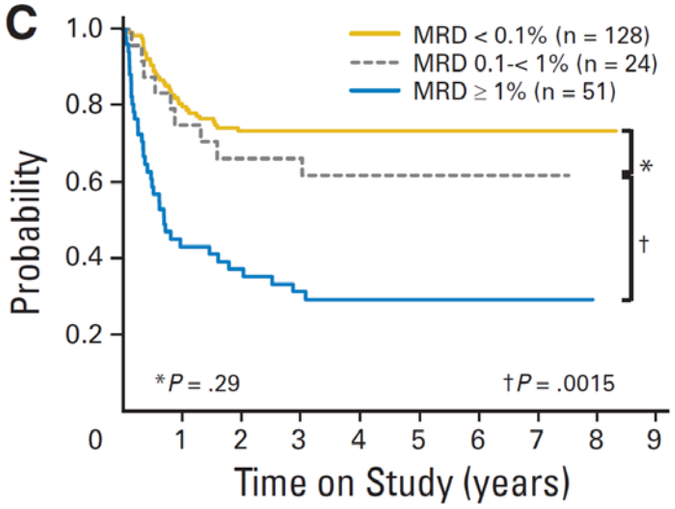
Figure 1. Cumulative incidence of relapse in children with ALL according to MRD levels at the end of remission induction. All patients were in complete morphologic remission at the time of the MRD studies. Levels of MRD were defined by the percentage of mononuclear cells expressing leukemia-specific immunophenotypes.

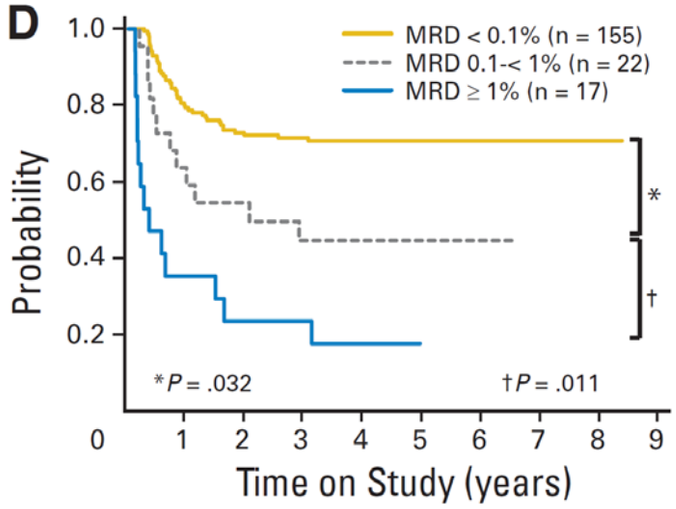
Coustan-Smith E et al. Blood 2000



Patel B et al. BJH 2009

EMR - LMA





JCO 2012

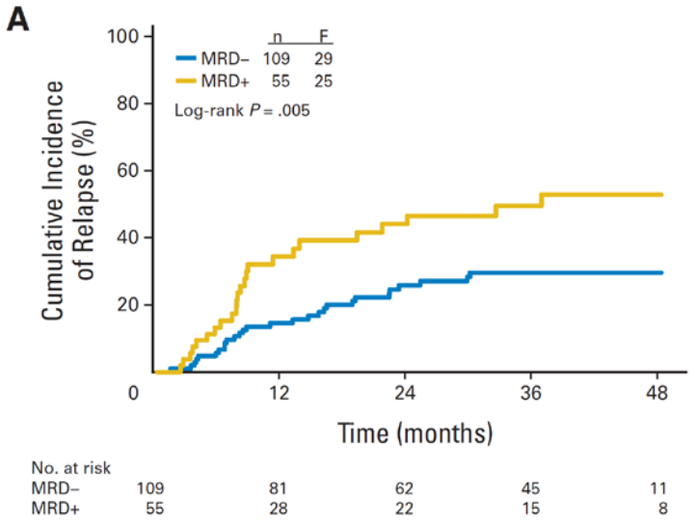
AML02

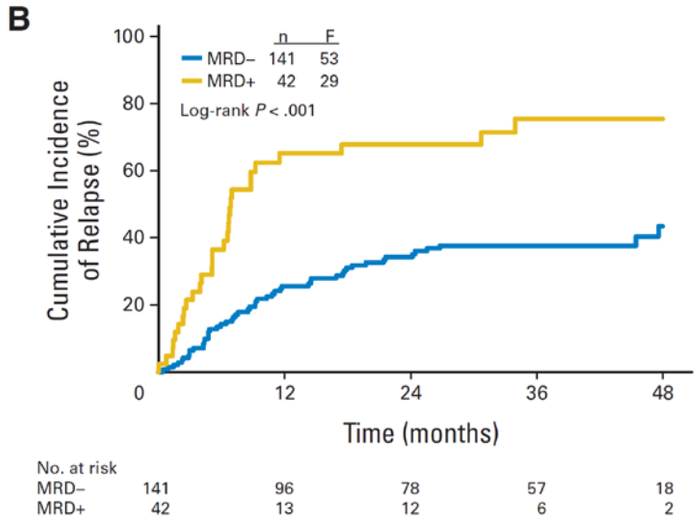
EFS 5 yr I-1 & I-2

≥ 1: 29,3

0.1-1: 61,8

< 0.1: 73,4





JCO 2013

HOVON-SAKK AML 42A

CIR I-1 & I-2

Cutoff: 0.1%

Vall d'Hebron

EMR - LMA

Table 6. Response criteria in AML

Category	Definition	Comment
Response		
CR without minimal residual disease (CR_{MRD-})	If studied pretreatment, CR with negativity for a genetic marker by RT-qPCR, or CR with negativity by MFC	Sensitivities vary by marker tested, and by method used; therefore, test used and sensitivity of the assay should be reported; analyses should be done in experienced laboratories (centralized diagnostics)
Complete remission (CR)	Bone marrow blasts <5%; absence of circulating blasts and blasts with Auer rods; absence of extramedullary disease; ANC $\geq 1.0 \times 10^9$/L (1000/μL); platelet count $\geq 100 \times 10^9$/L (100 000/μL)	MRD^+ or unknown
CR with incomplete hematologic recovery (CR_i)	All CR criteria except for residual neutropenia ($<1.0 \times 10^9$/L [1000/μL]) or thrombocytopenia ($<100 \times 10^9$/L [100 000/μL])	
Morphologic leukemia-free state (MLFS)	Bone marrow blasts <5%; absence of blasts with Auer rods; absence of extramedullary disease; no hematologic recovery required	Marrow should not merely be "aplastic"; at least 200 cells should be enumerated or cellularity should be at least 10%
Partial remission (PR)	All hematologic criteria of CR; decrease of bone marrow blast percentage to 5% to 25%; and decrease of pretreatment bone marrow blast percentage by at least 50%	Especially important in the context of phase 1-2 clinical trials

ELN AML Recommendations. Blood 2017

EMR - LMA

Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
LMA1	CD11b	CD15	CD16	CD117	CD33	CD13	CD34	CD10	HLA-DR	CD45
LMA2	CD35	CD300e	CD64	CD117	CD14	CD7	CD34	CD56	HLA-DR	CD45
LMA3	CD71	CD105	CD38	CD117	CD33	CD36	CD34	CD235a	HLA-DR	CD45
LMA4	CD61+41	NG2	CD19	CD117	CD25	CD2	CD34	CD9	HLA-DR	CD45
LMA5	CD141	CD203c	CD4	CD117	CD11c	CD123	CD34	CD56	HLA-DR	CD45

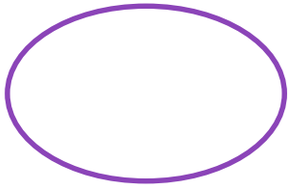
LEUKEMIA ASSOCIATED IMMUNOPHENOTYPES

"LAIPs"

AML with t(8;21)(q22;q22); RUNX1-RUNX1T1

Diagnosis

Blasts:
82.0%



Leukemic blasts

Follow-up

Post-induction

MRD: 0.033%

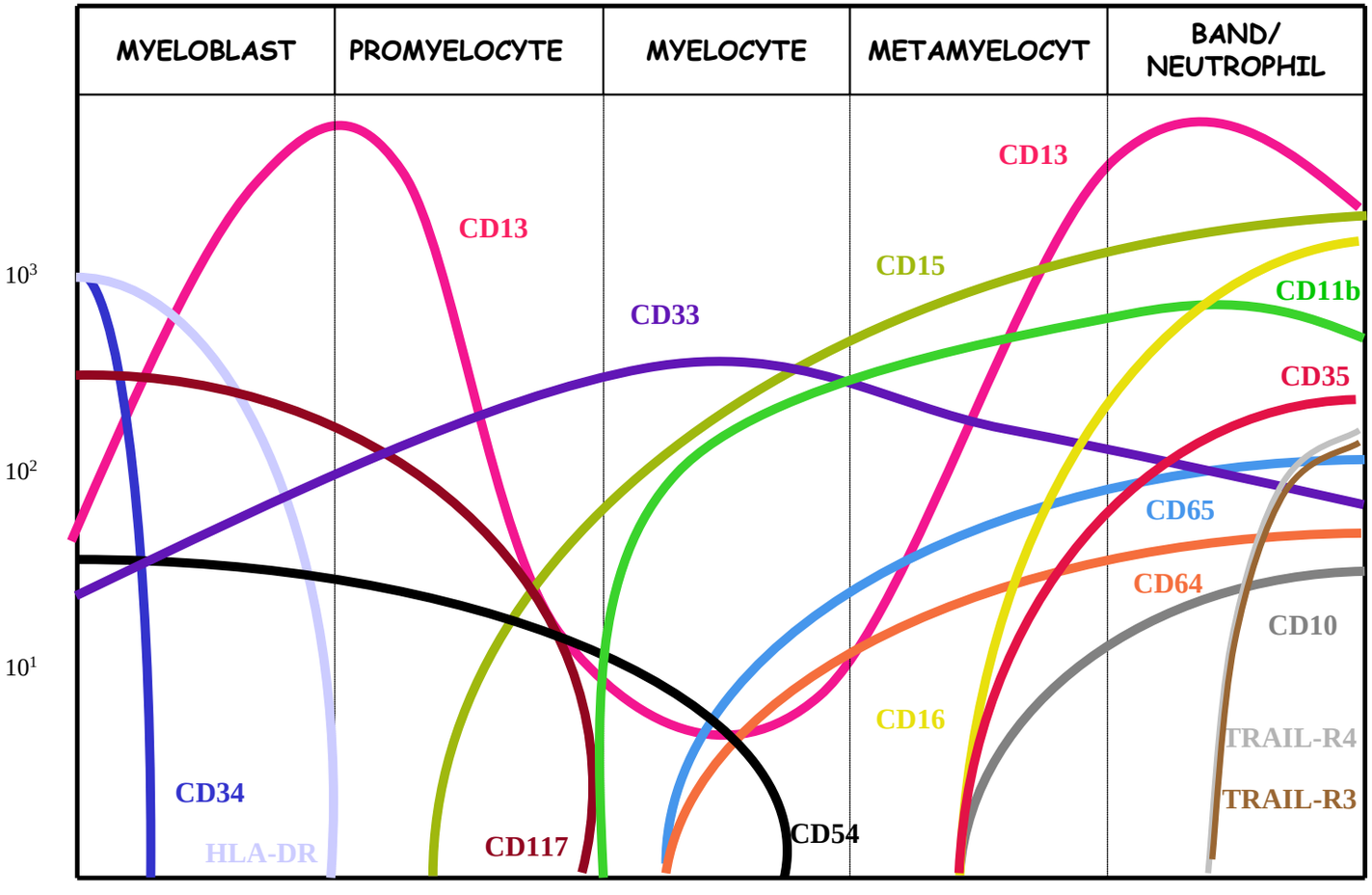
Post-consolidation

MRD: neg

45,X,-Y, t(8;21)(q22;q22)[1]/ 65-85,XXY,-Y,-1,-2,-5,t(8;21)(q22;q22),-11,-14, -16[17]/ 46,XY[2]

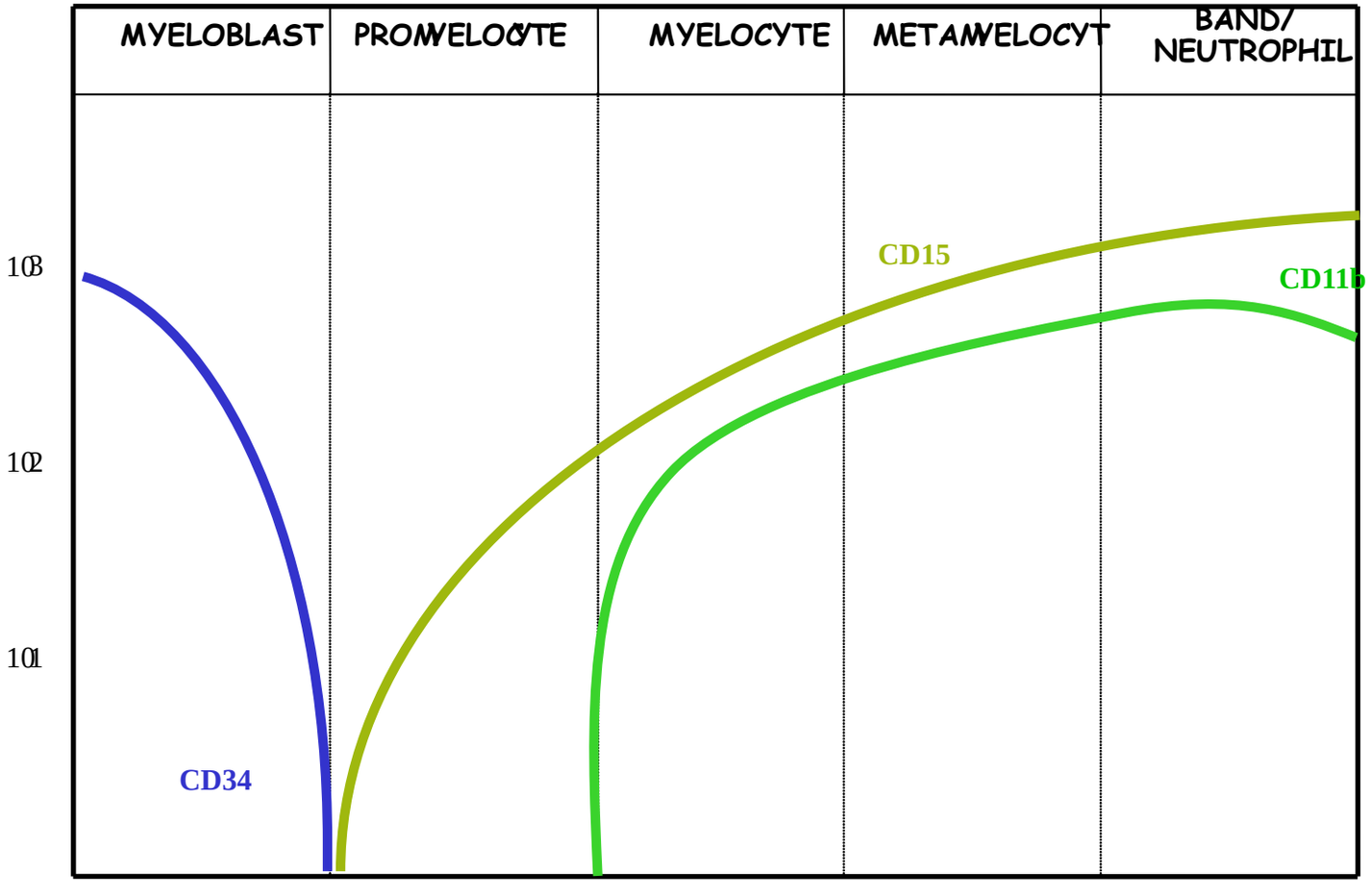
EMR - DfN

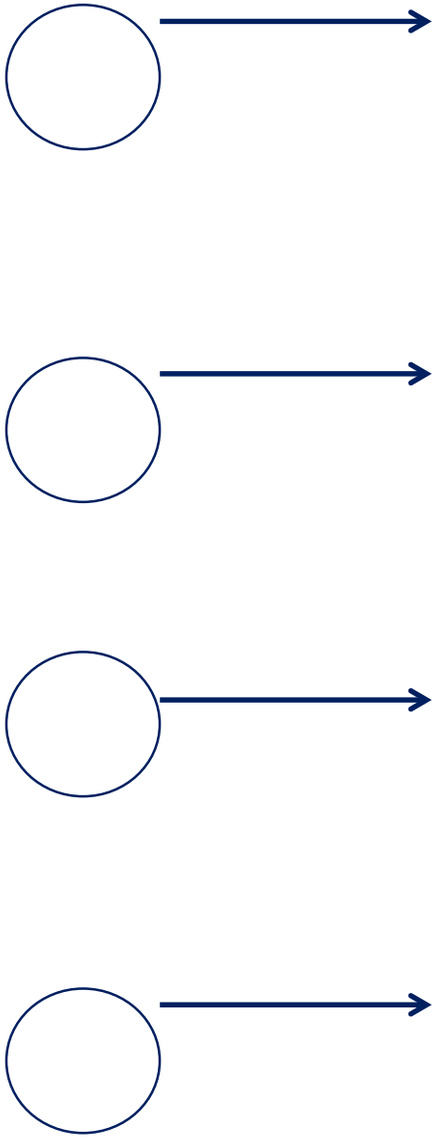
Maduración mieloide normal



EMR - DfN

Maduración mieloide normal





Diagnosis

Blasts:
14.3%

Post-induction

MRD: 2.19%

Post-consolidation

MRD: neg

+ 1 yr

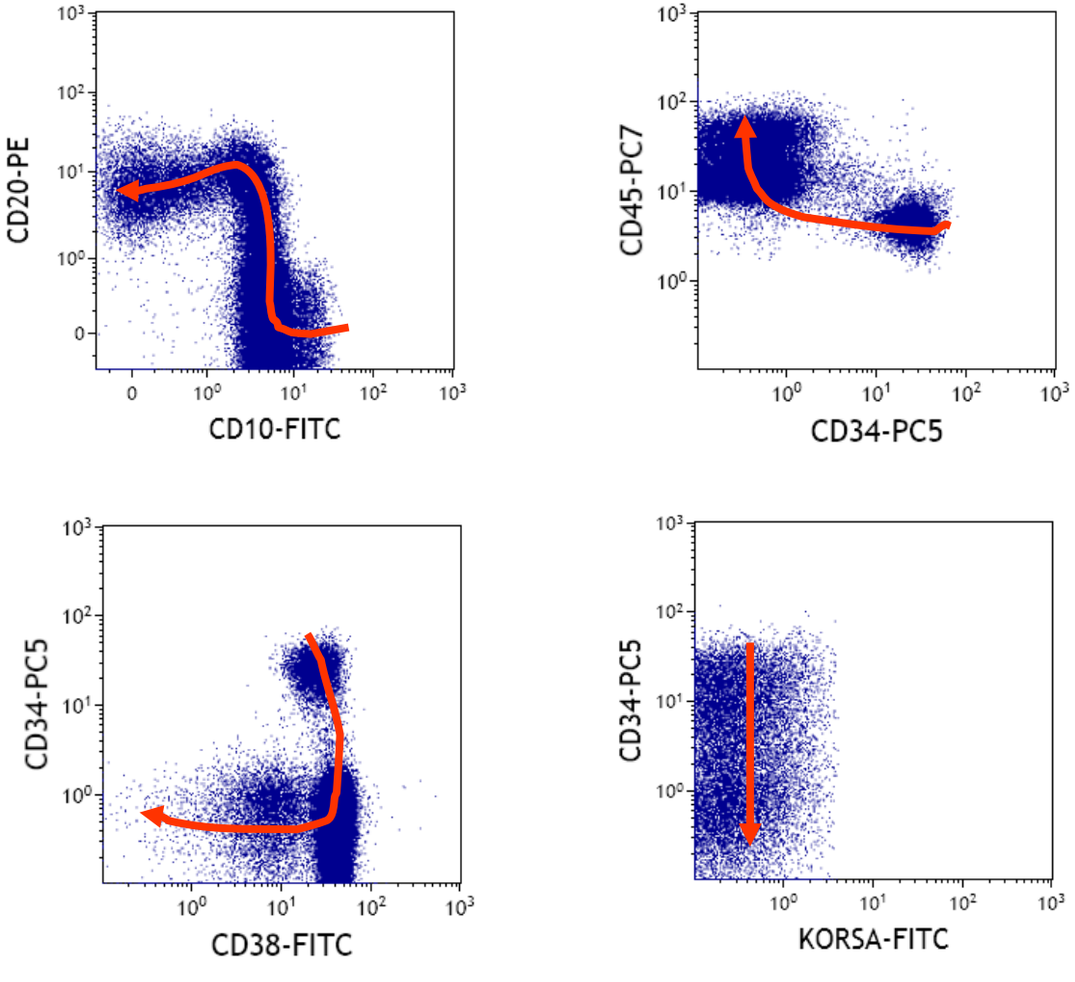
MRD: 0.498%

AML with inv(16)(p13.1q22)/ CBFβ-MYH11

Vall d'Hebron

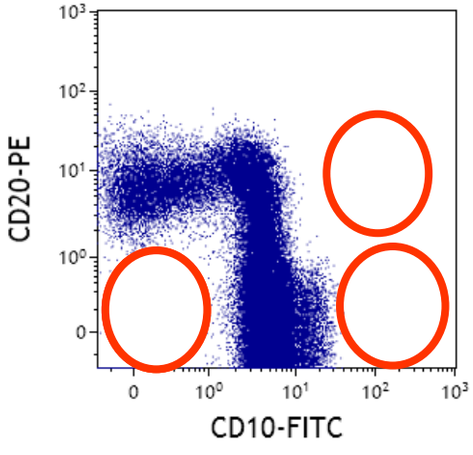
EMR - DfN

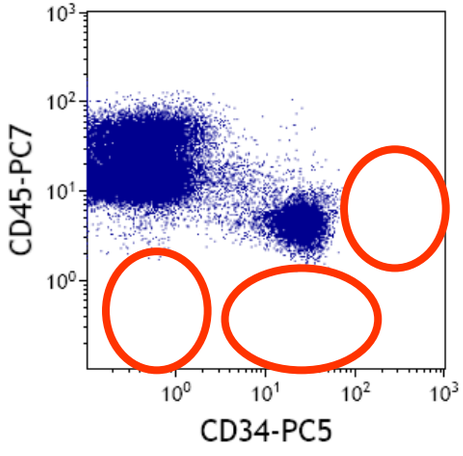
Maduración B normal

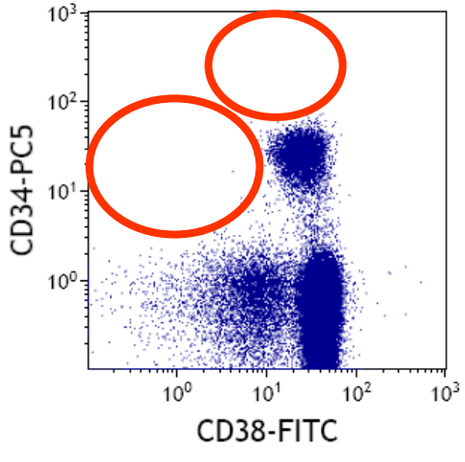


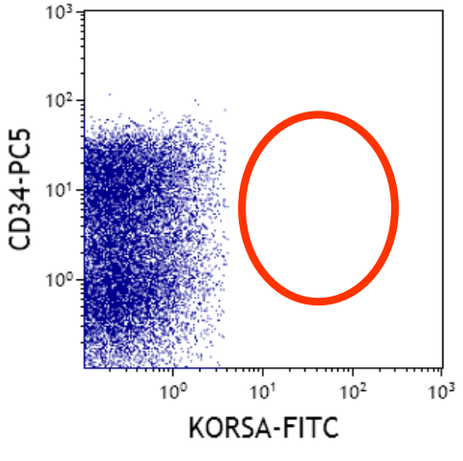
EMR - DfN

Empty spaces







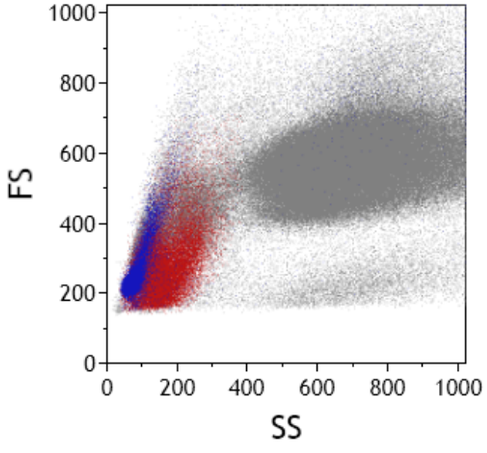


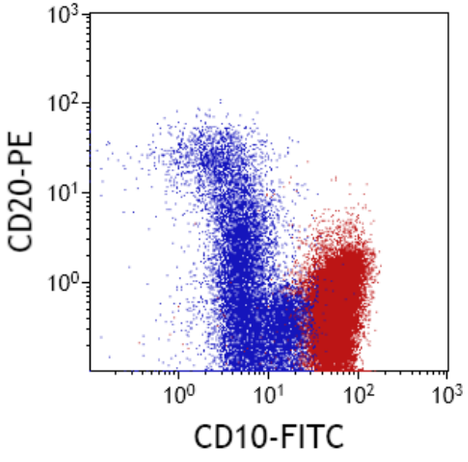
Hematopoyesis B normal

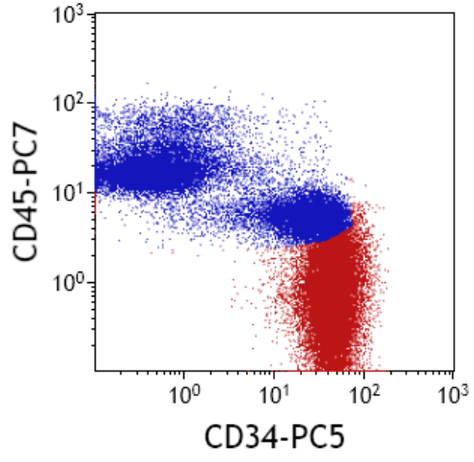
Linfoblastos B leucémicos

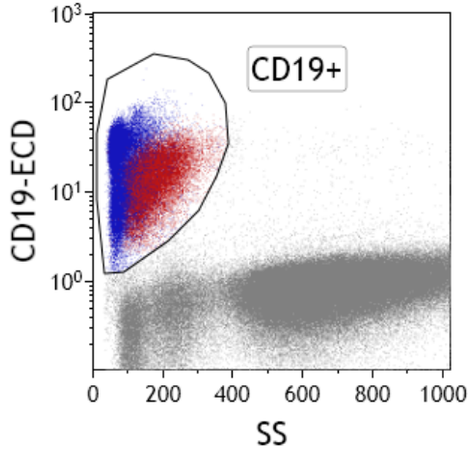
Vall d'Hebron

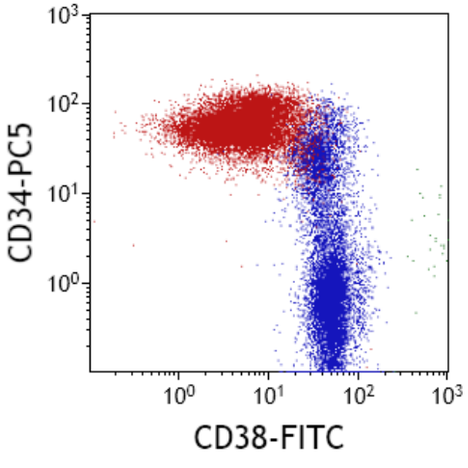
LLA-B Ph+ blastos: 11,04%

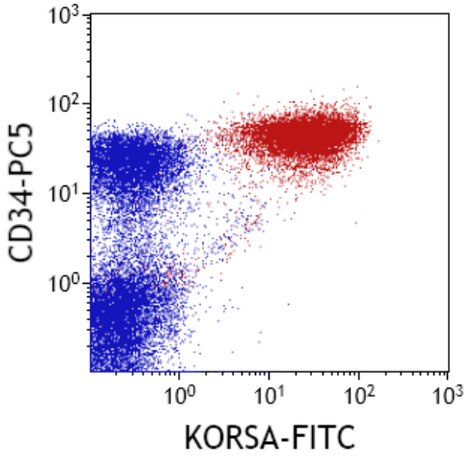










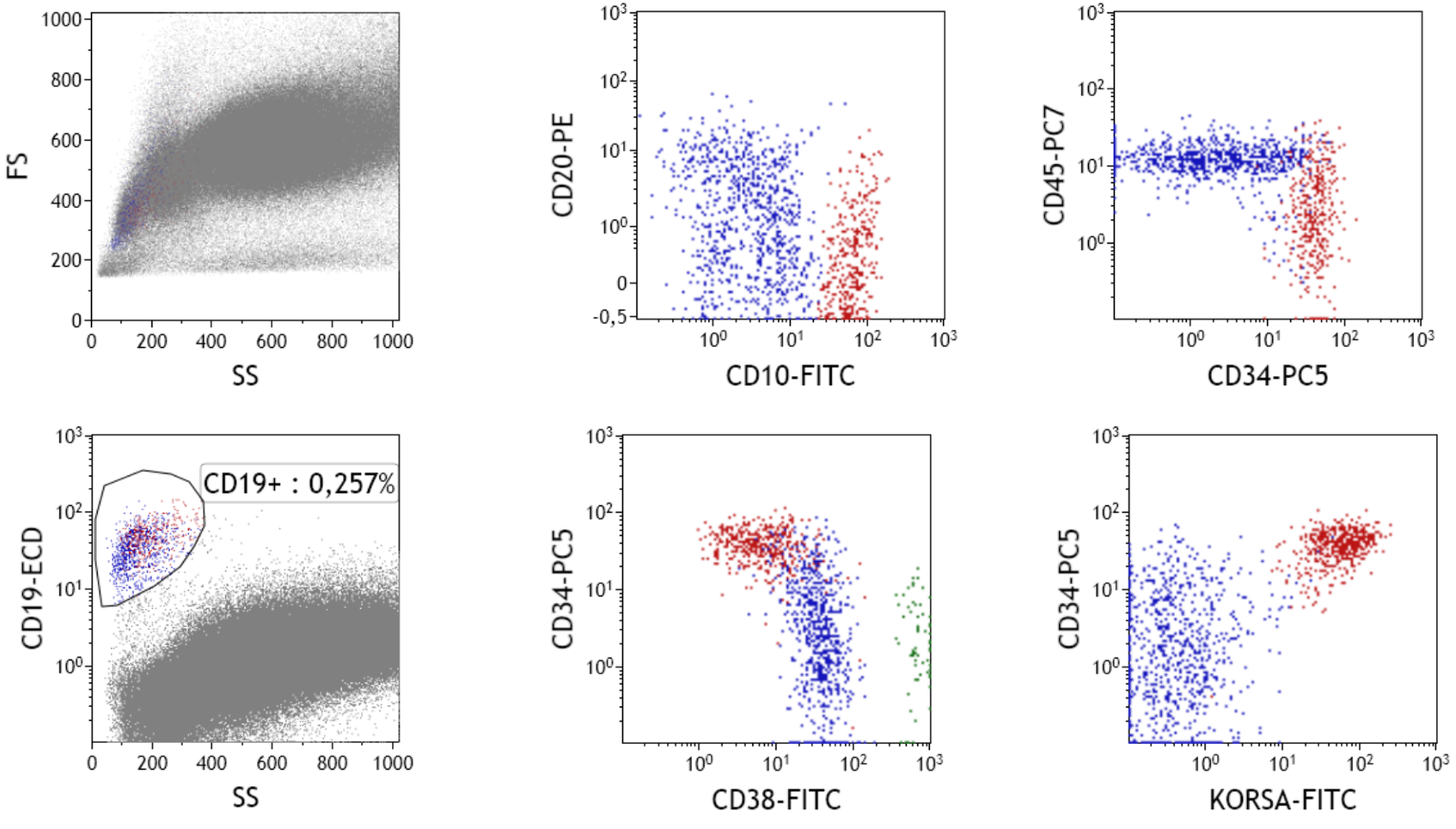


Hematopoyesis B normal

Linfoblastos B leucémicos

Células plasmáticas

LLA-B Ph+ EMR: 0,056%



Hematopoyesis B normal

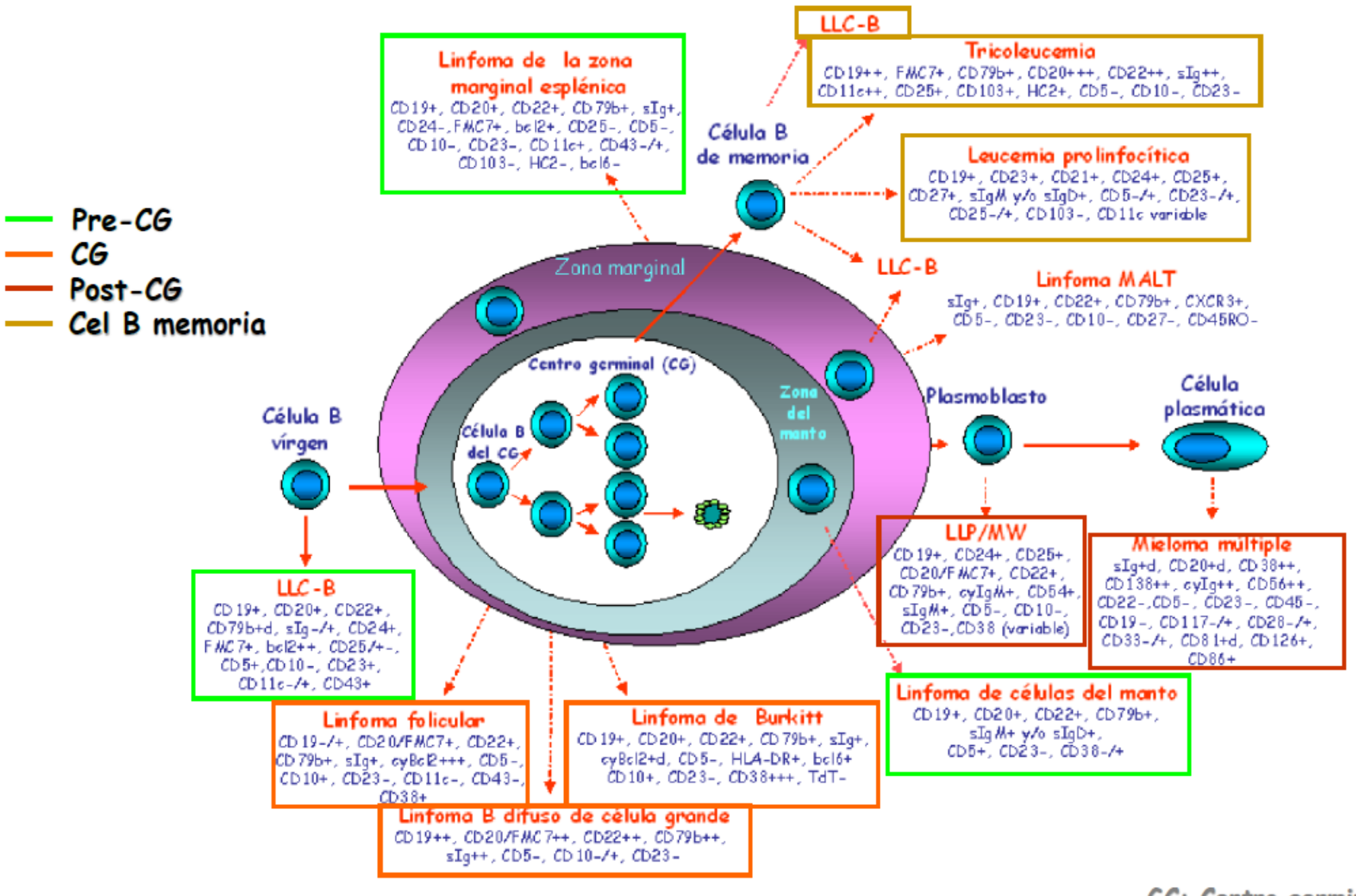
Linfoblastos B leucémicos

Células plasmáticas

Panel 12. Composition of BCP-ALL MRD panel and technical information on reagents*

Tube	PacB	PacO	FITC	PE	PerCPCy5.5	PECy7	APC	APCA750
1	CD20	CD45	CD81	CD66c + CD123	CD34	CD19	CD10	CD38
2	CD20	CD45	CD81	CD304 + CD73	CD34	CD19	CD10	CD38

Origen de los síndromes linfoproliferativos B


Pre-CG
CG
Post-CG
Cel B memoria
Linfoma de la zona marginal esplénica
CD19+, CD20+, CD22+, CD79b+, sIg+, CD24-, FMC7+, bcl2+, CD25-, CD5-, CD10-, CD23-, CD11c+, CD43-/+, CD103-, HC2-, bcl6-
LLC-B
Tricoleucemia
CD19++, FMC7+, CD79b+, CD20+++, CD22++, sIg++, CD11c++, CD25+, CD103+, HC2+, CD5-, CD10-, CD23-
Célula B de memoria
Leucemia prolinfocítica
CD19+, CD23+, CD21+, CD24+, CD25+, CD27+, sIgM y/o sIgD+, CD5-/+, CD23-/+, CD25-/+, CD103-, CD11c variable
LLC-B
Linfoma MALT
sIg+, CD19+, CD22+, CD79b+, CXCR3+, CD5-, CD23-, CD10-, CD27-, CD45RO-
Zona marginal
Centro germinal (CG)
Célula B del CG
Zona del manto
Célula B vírgen
Plasmoblasto
Célula plasmática
LLP/MW
CD19+, CD24+, CD25+, CD20/FMC7+, CD22+, CD79b+, cyIgM+, CD54+, sIgM+, CD5-, CD10-, CD23-, CD38 (variable)
Mieloma múltiple
sIg+d, CD20+d, CD38++, CD138++, cyIg++, CD56++, CD22-, CD5-, CD23-, CD45-, CD19-, CD117-/+, CD28-/+, CD33-/+, CD81+d, CD126+, CD86+
LLC-B
CD19+, CD20+, CD22+, CD79b+d, sIg-/+, CD24+, FMC7+, bcl2++, CD25/+-, CD5+, CD10-, CD23+, CD11c-/+, CD43+
Linfoma de células del manto
CD19+, CD20+, CD22+, CD79b+, sIgM+ y/o sIgD+, CD5+, CD23-, CD38-/+
Linfoma folicular
CD19-/+, CD20/FMC7+, CD22+, CD79b+, sIg+, cyBcl2+++, CD5-, CD10+, CD23-, CD11c-, CD43-, CD38+
Linfoma de Burkitt
CD19+, CD20+, CD22+, CD79b+, sIg+, cyBcl2+d, CD5-, HLA-DR+, bcl6+ CD10+, CD23-, CD38+++, TdT-
Linfoma B difuso de célula grande
CD19++, CD20/FMC7++, CD22++, CD79b++, sIg++, CD5-, CD10-/+, CD23-

GC: Centro germinal

Caracterización de los SLP-B

Panel de estudio "B"

Marcadores "obligados"

Gating marker: CD19

Panel básico: CD5, CD20, CD22, CD23, CD103, FMC7, CD10, CD25, CD79b, CD38, kappa, lambda

Marcadores "opcionales"

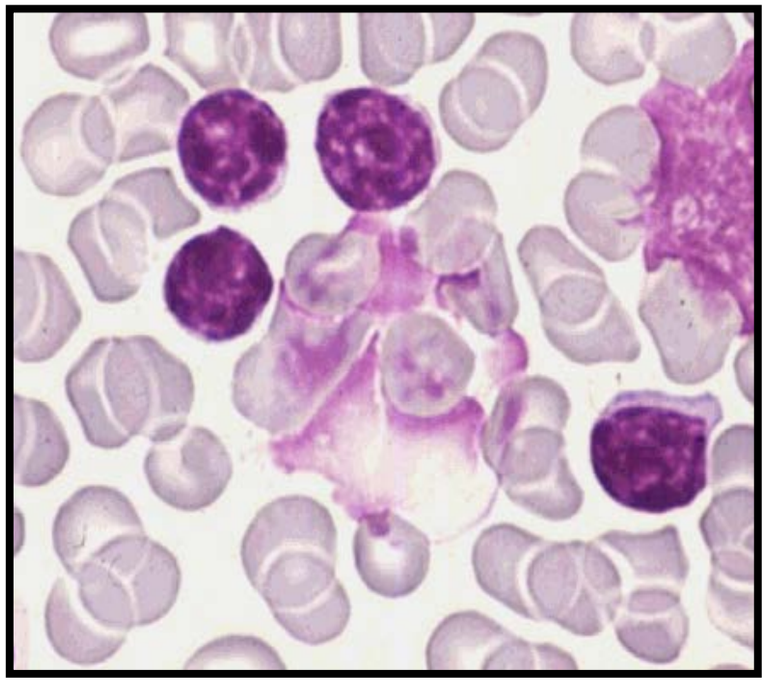
CD2, CD7, CD123, CD138, HLA-DR, CD24, CD43, CD81, IgG, IgA, IgM, IgD. Citoplasmáticos: Bcl2, ZAP-70

Recomendaciones del grupo ELN (European Leukemia Net), 2005

LLC - Morfología

LLC típica

- ✓ Linfocito B maduro
- ✓ Tamaño pequeño
- ✓ Frágil (sombras de Gumprecht)
- ✓ Citoplasma escaso
- ✓ Cromatina densa sin nucleolos
- ✓ Fenotipo B CD5+, IgM+, IgD+/-
- ✓ Reordenamiento clonal de IgH



LLC - Morfología

✓ **LLC/ LP**

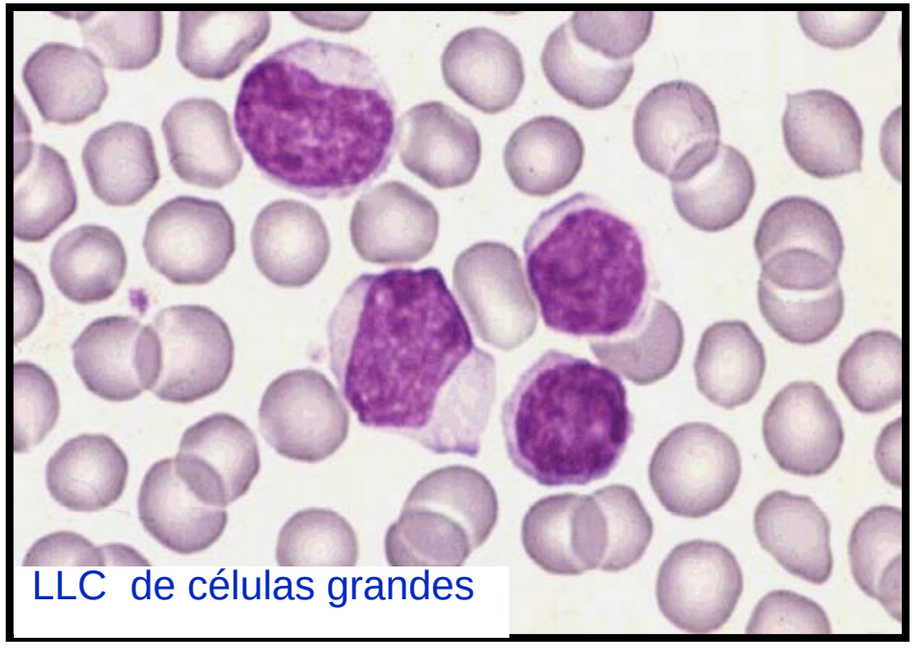
Prolinfocitos 11-55%

✓ **LLC variante 'estimulada' (mixta o de células grandes)**

Células grandes hiperbasófilas, prolinfocitos ≤ 10%, centrocitos

✓ **LLC con centrocitos** > 10%

✓ **LLC parainmunoblástica** (muy infrecuente)



LLC de células grandes

Síndromes linfoproliferativos B

Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF750	PaB	KrO
SLP-S	Kappa + CD8	Lambda + CD4	CD19	CD56			CD3	CD20	CD45
SLPB1	CD22	CD23	CD19	CD79b	CD200	CD5	CD43	CD20	CD45
SLPB2	CD103	CD305 (LAIR1)	CD19	CD11c	CD25	CD123	CD27	FMC7	CD45
SLPB3	CD81	ROR1	CD19	CD38	CD10	CD5	CD44	CD20	CD45
ZAP	CD49d	Cit-ZAP70	CD19	CD56		CD5	CD3		CD45

FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin; ECD, Phycoerythrin-Texas red; PC5.5, Phycoerythrin-cyanin 5.5; PC7, Phycoerythrin-cyanin 7; APC, Allophycocyanin; AAF700, APC-Alexa Fluor 700; AAF750, APC- Alexa Fluor 750; PaB, Pacific Blue; KrO, Khrome Orange

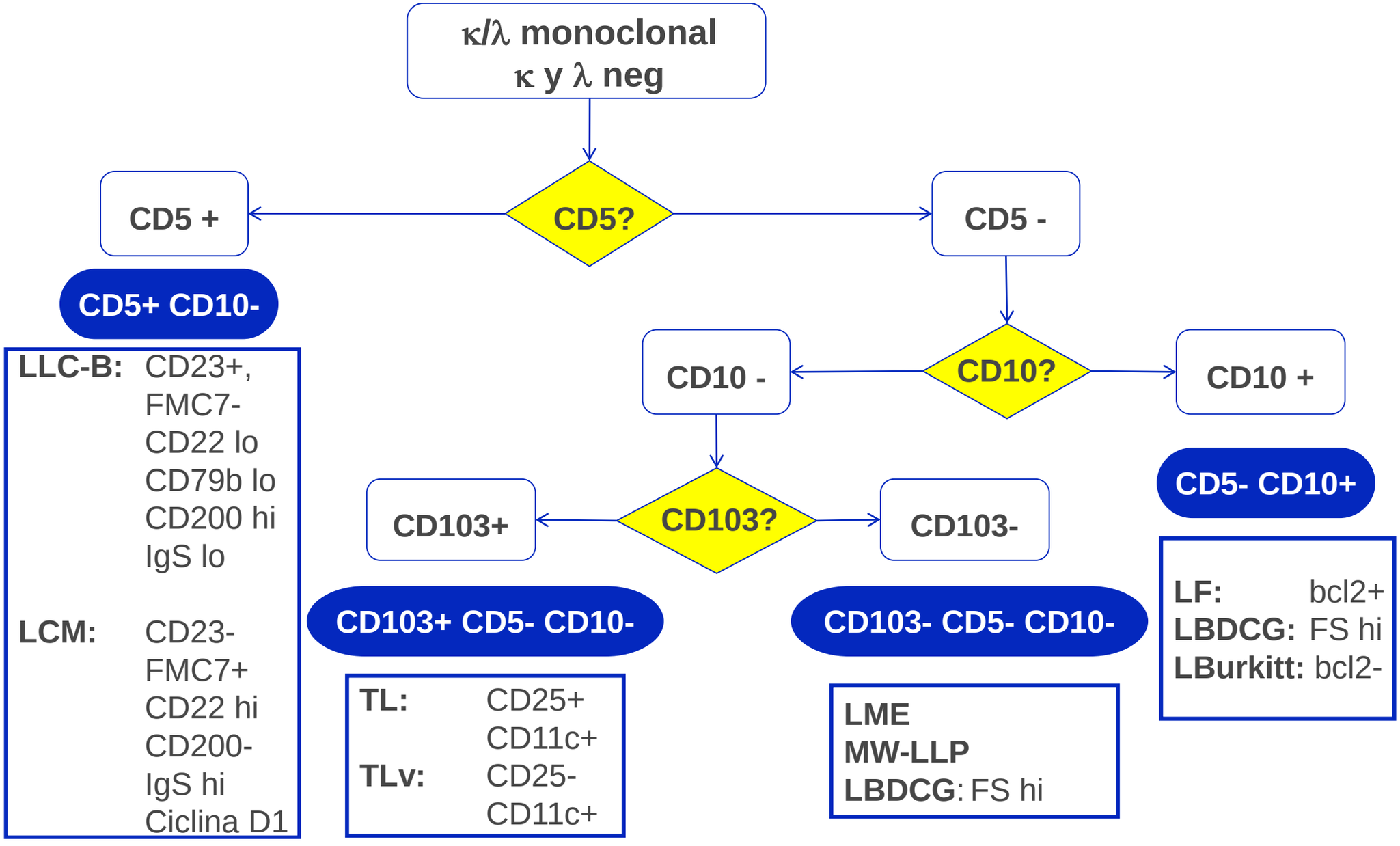
Síndromes linfoproliferativos B

SLP	CD19	CD79b	CD5	CD23	CD10	FMC7	CD103	CD25	IgS
LF	++	++	-	-/+	++	++	-	-	F
LCM	++	++	++	-	-	++	-	-/+	F
LLC-B	++	-/+	++	++	-	-	-	-	D
LPL-B	++	++	-/+	-	-	++	-	-	F
TL	++	-/+	-	-	-	++	++	++	F
TLv	++	+	-	-	-	++	++	-	F
LME	++	++	-/+	-/+	-	++	-	-/+	F
LBDCG	++	++	-	-/+	+	++	-	-	F

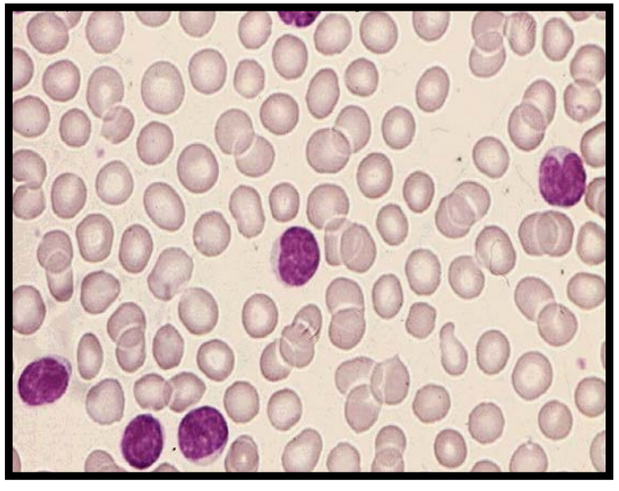
LF, linfoma folicular; LCM, linfoma de células del manto; LLC-B, leucemia linfocítica crónica B; LPL-B, leucemia prolinfocítica B; TL, tricoleucemia; TLv, tricoleucemia variante; LME, linfoma marginal esplénico; LBDCG, linfoma B difuso de célula grande;

(F) intensidad fuerte; (D) Intensidad débil; (++) positivo en >75% de los casos; (+) positivo entre el 25 y el 75%; (-/+) positivo entre el 10 y el 25%; (-) negativo o positivo en < 10% de los casos.

SLP-B algoritmo diagnóstico



LLC - *score*



L. Folicular

LLC/LPL

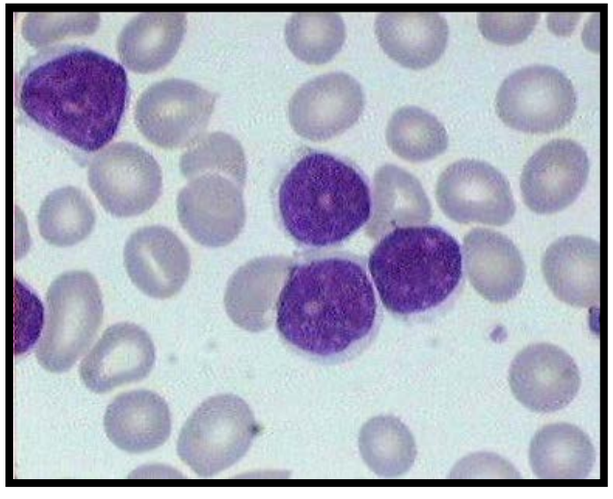
Sistema de puntuación

	0	1
CD5	-	+
CD23	-	+
FMC7	+	-
CD79b	intenso	-/ débil
IgS	intensa	-/ débil

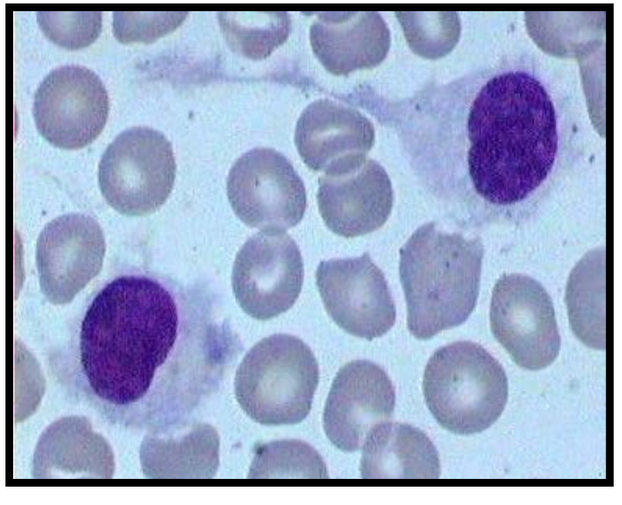
4 - 5: IF típico. 96,8% *"accuracy"*

3: IF atípico

Leukemia 1994; 8(10):1640-5
Am J Clin Pathol 1997; 108(4):378-82

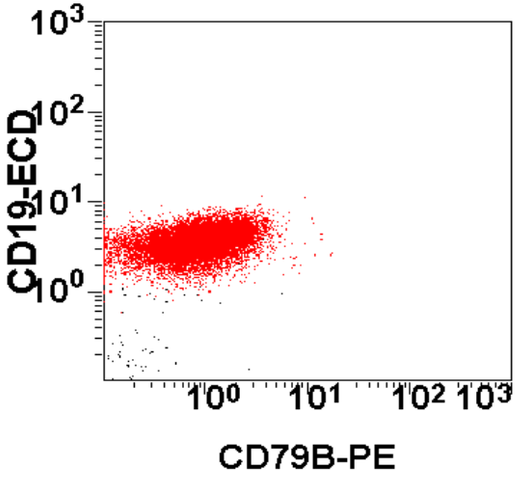


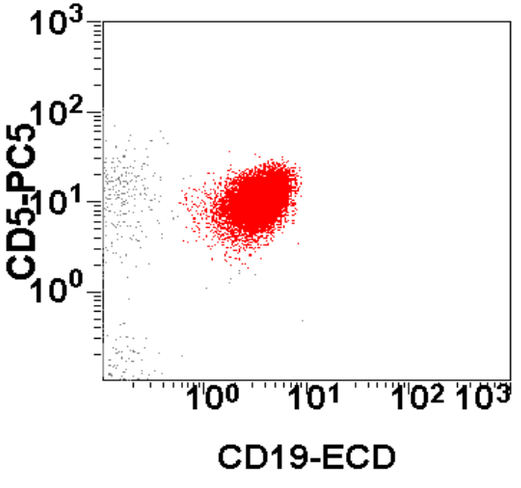
L. Prolinfocítica

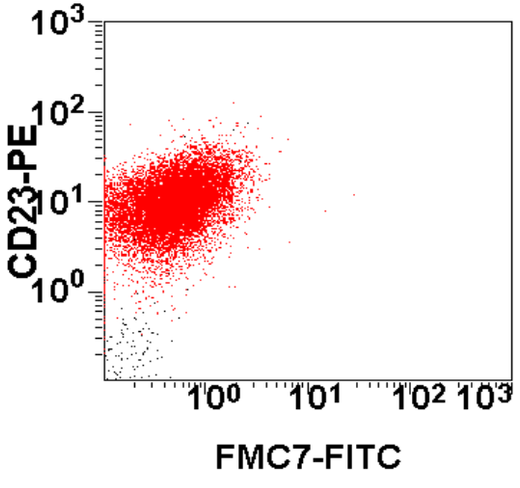


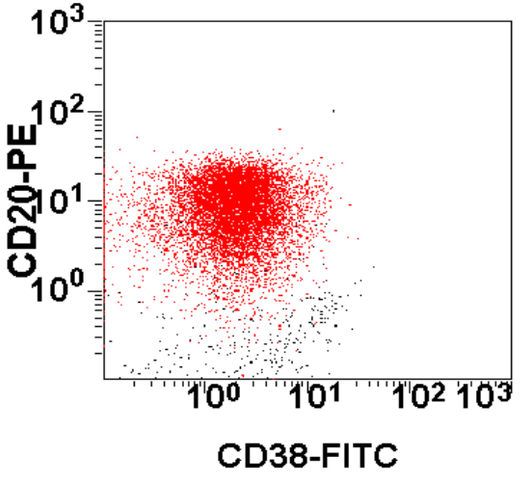
Tricoleucemia

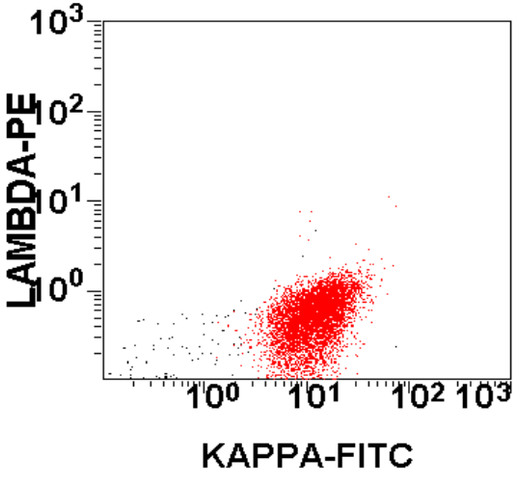
LLC típica

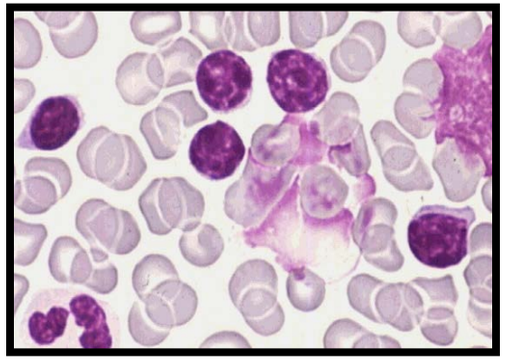






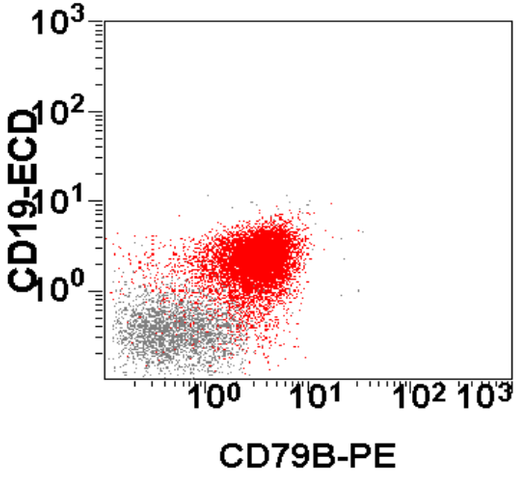


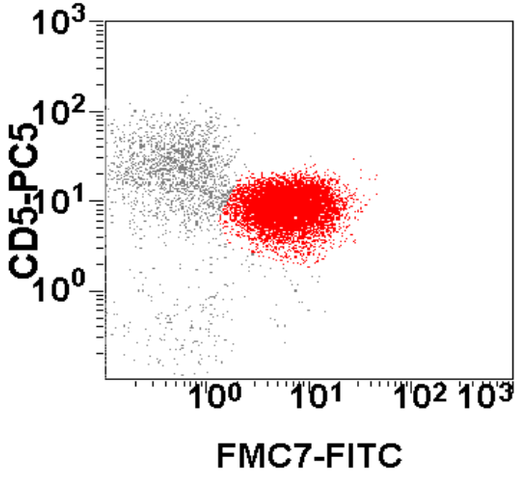


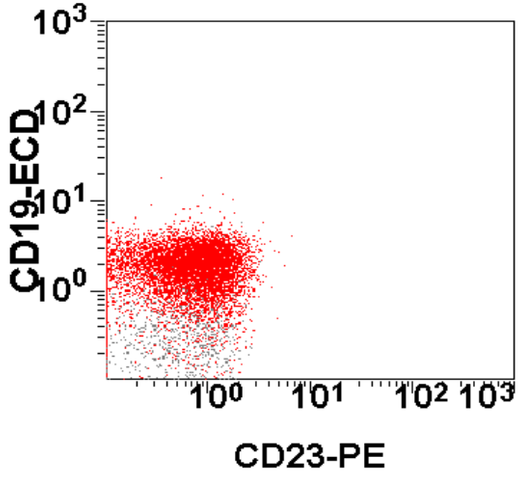


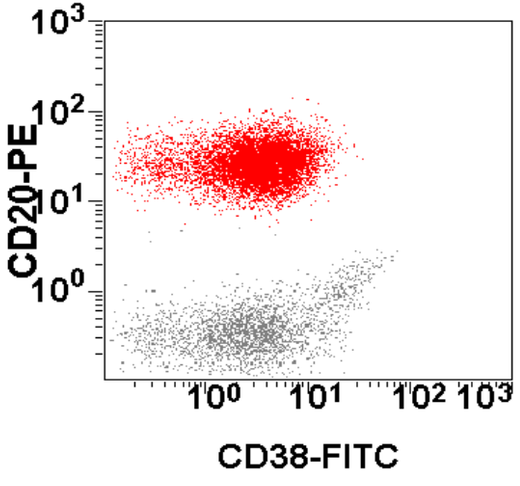
S.P. (*score* 4/5): CD19+, CD5+, FMC7-, CD23-, IgS++, CD79b+, CD20++

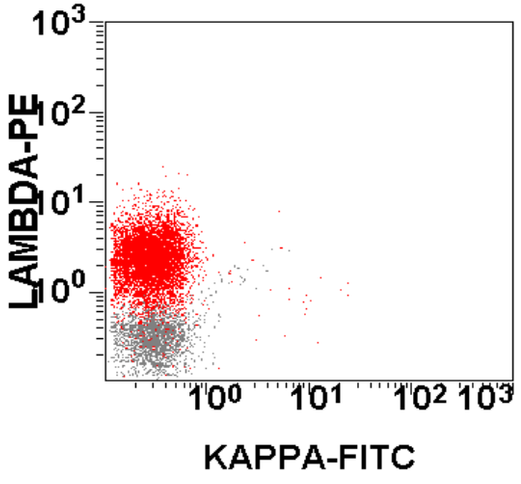
Linfoma de células del manto

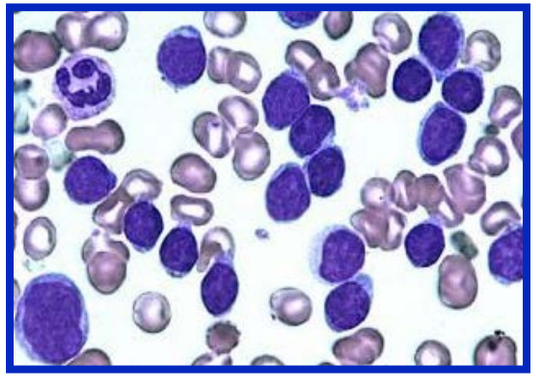






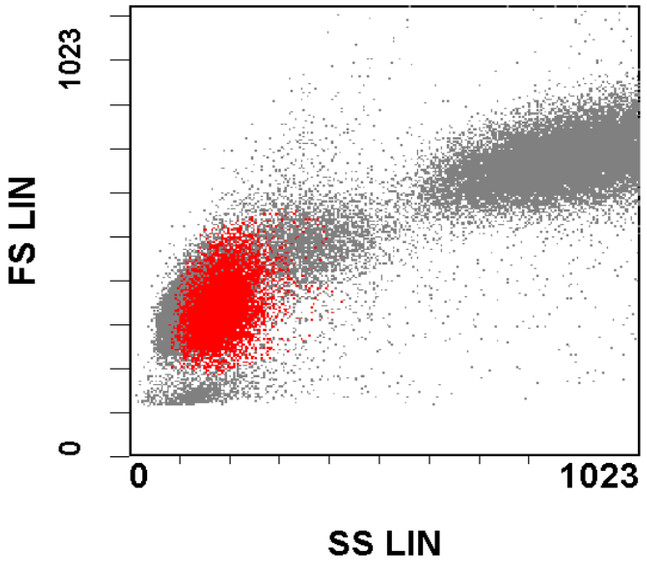


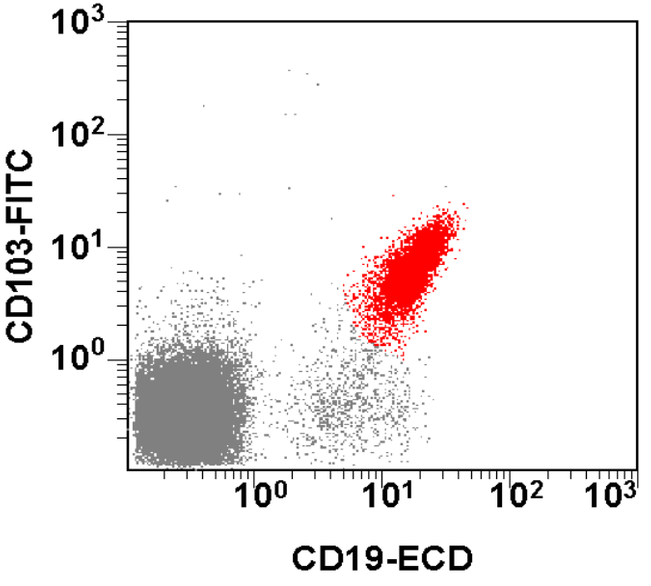


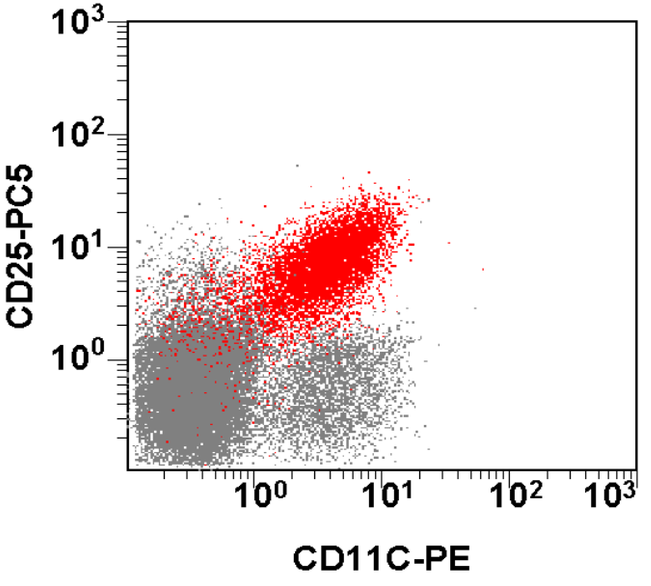


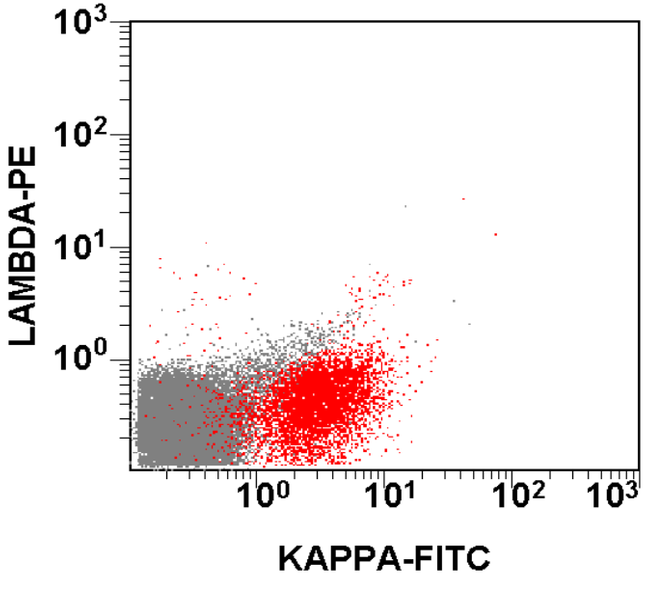
sangre (*score* 1 de 5): CD19+, CD5+, FMC7+, CD23-, IgS++, CD79b++, CD20+++

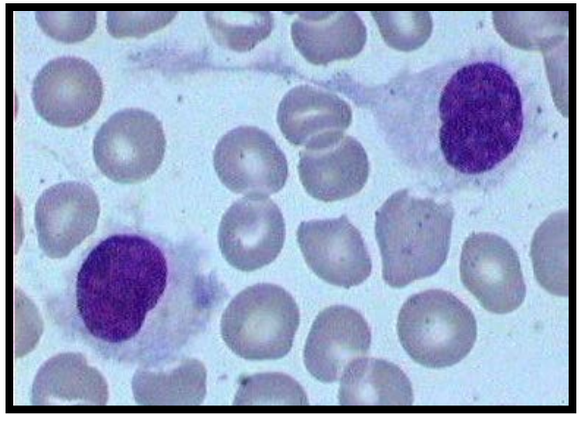
Tricoleucemia









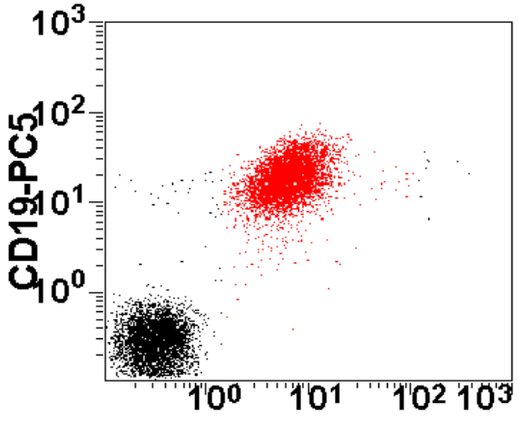


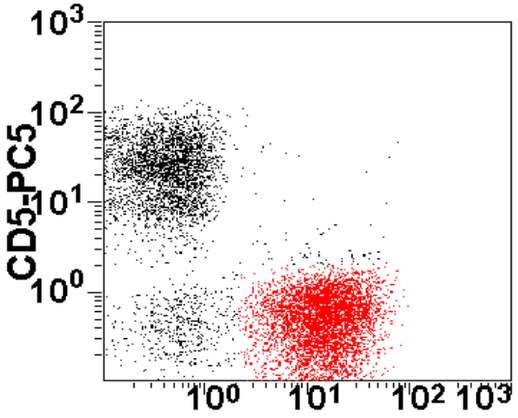
Sangre periférica

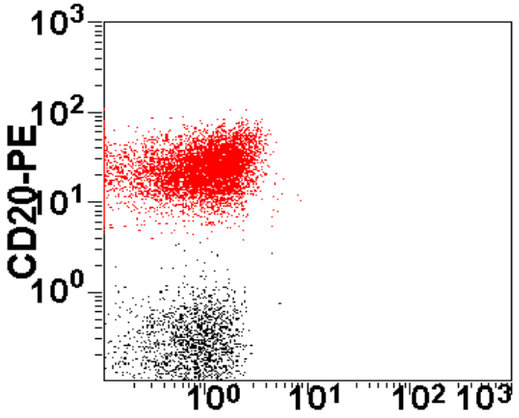
CD19+, CD5-, CD10-, FMC7++, CD11c+++, CD103+, IgS+

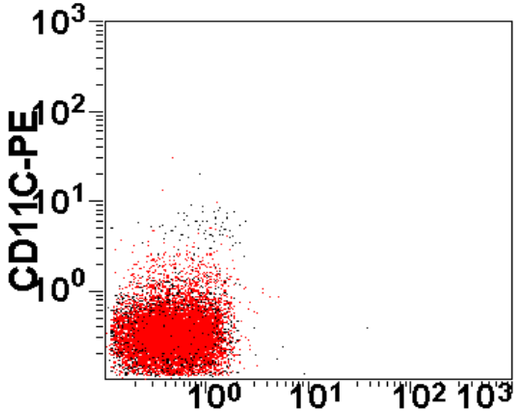
Vall d'Hebron

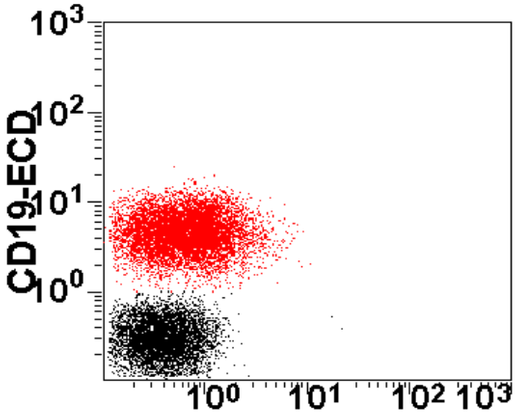
Linfoma marginal esplénico









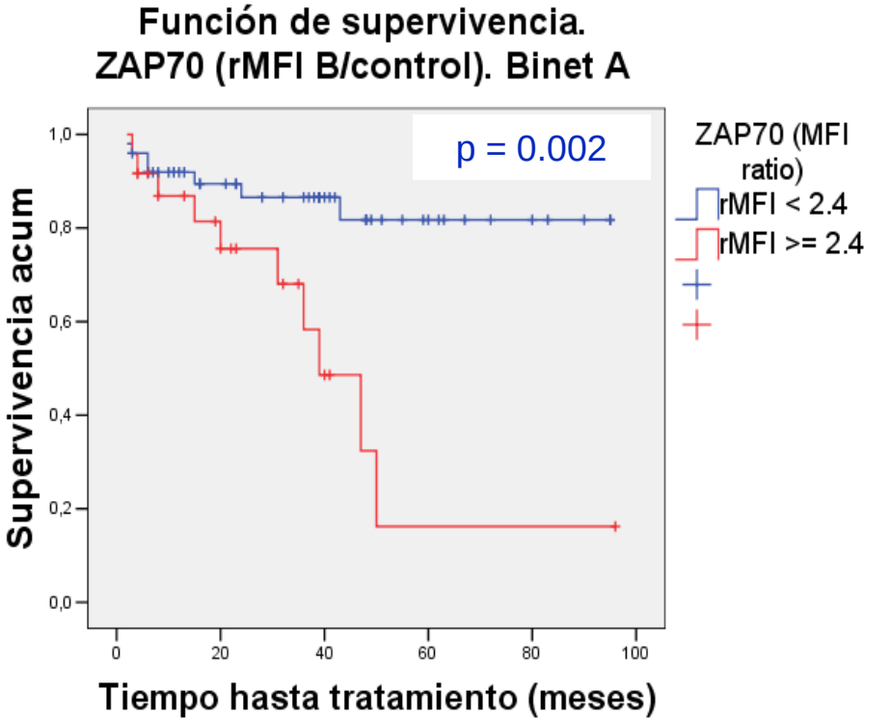


Sangre periférica

CD19dim, CD5-, CD10-, FMC7++, CD23-, IgS+++, CD20+++

LLC – ZAP70

Supervivencia libre de progresión (PFS)



rMFI < 2.4

Mediana PFS: NA

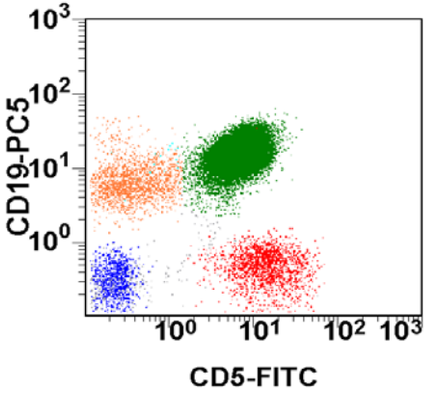
•a 200 m * : 15 años

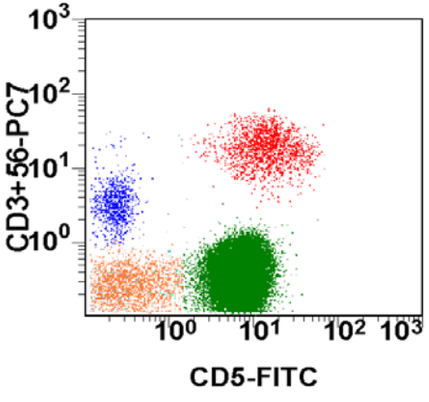
rMFI ≥ 2.4

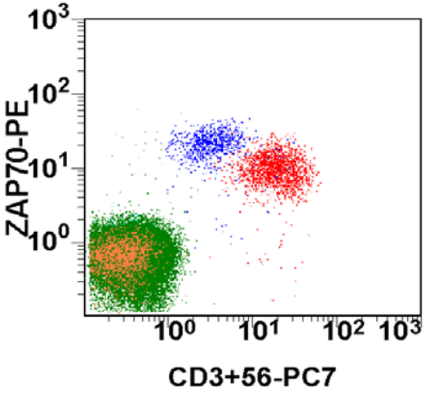
Mediana PFS: 39 m (IC 95%: 27.2 a 50.8 m)

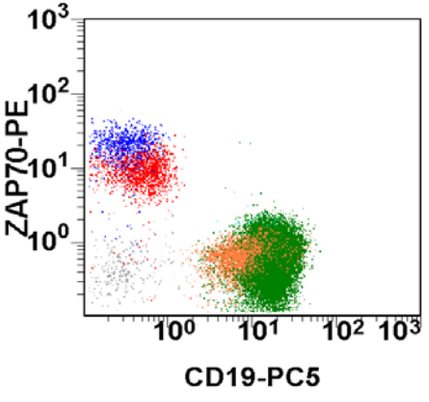
Percentil 75%: 50 m (IC 95%: 47.2 a 55.2 m)

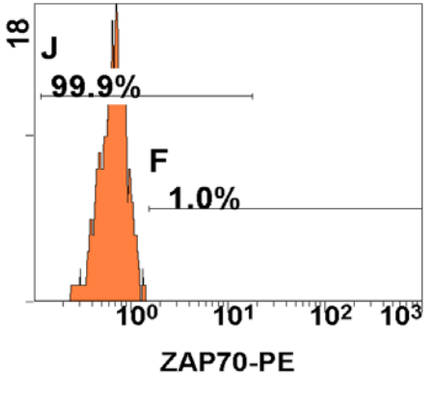
LLC – ZAP70

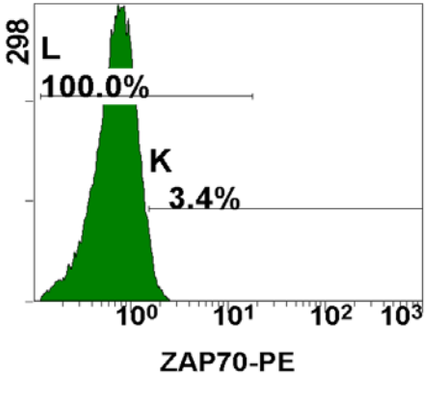


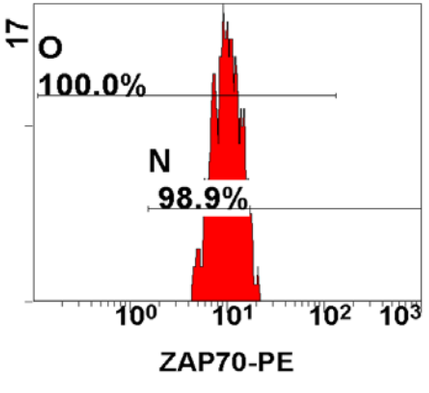


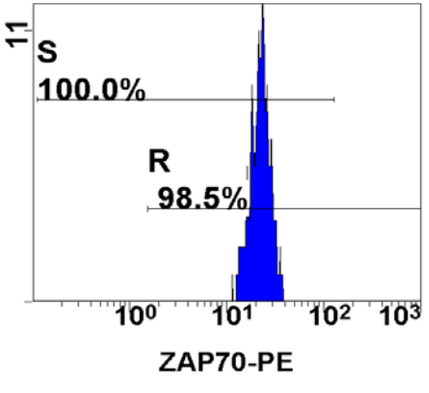












Ly B normales
CD19^{+}, CD5^{-}

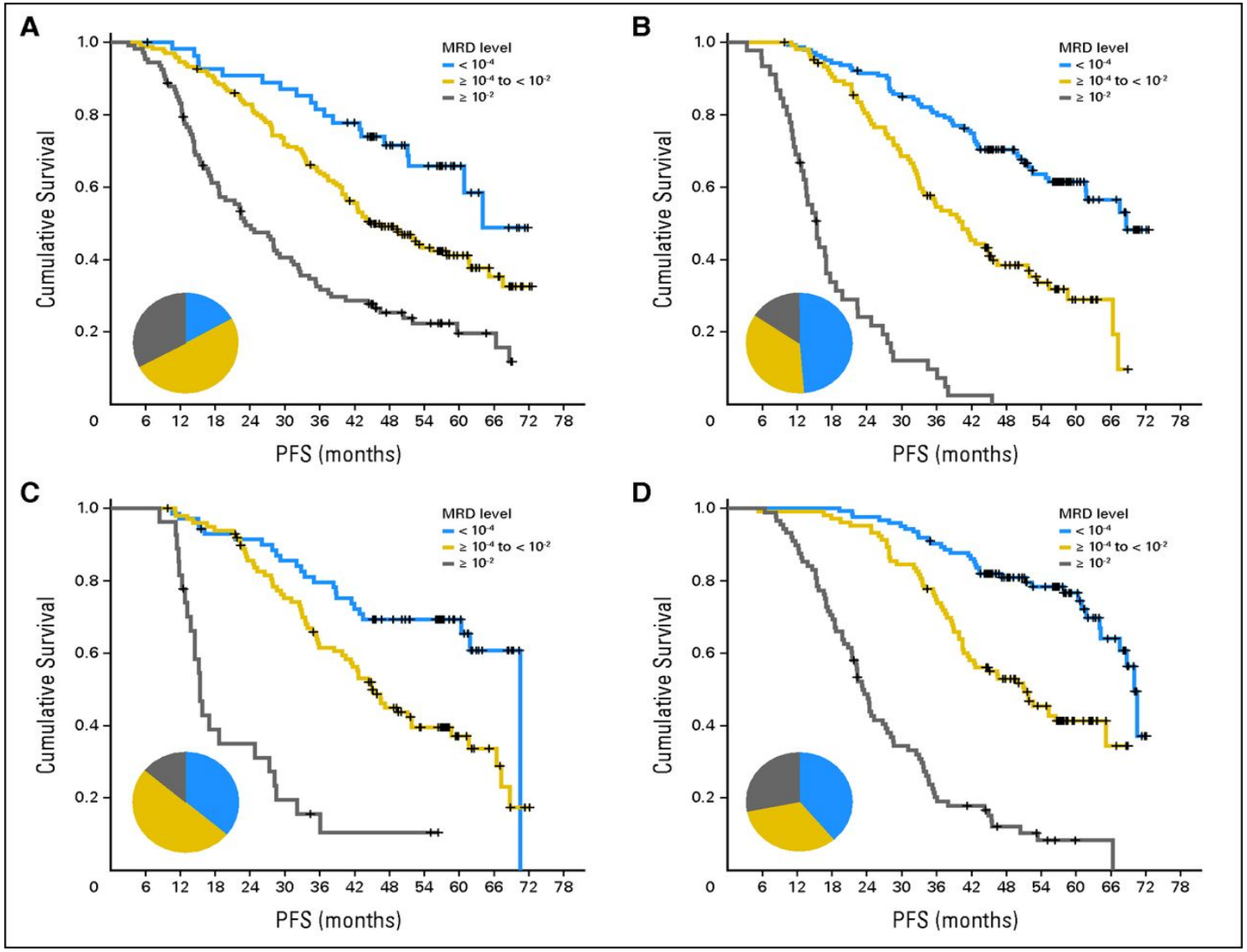
Ly B LLC-B CD19^{+}, CD5^{+}

Ly T
CD3+56^{+}, CD5^{+}

Ly NK
CD3+56^{+}, CD5^{-}

Vall d'Hebron

PFS in patients with CLL grouped by MRD levels assessed (A) in PB at interim staging, (B) in PB at final restaging, (C) in bone marrow at final restaging, and (D) in PB during follow-up



Sebastian Böttcher et al. JCO 2012;30:980-988

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LLC - EMR

Leukemia (2016) 30, 929–936
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www.nature.com/leu

ORIGINAL ARTICLE

A complementary role of multiparameter flow cytometry and high-throughput sequencing for minimal residual disease detection in chronic lymphocytic leukemia: an European Research Initiative on CLL study

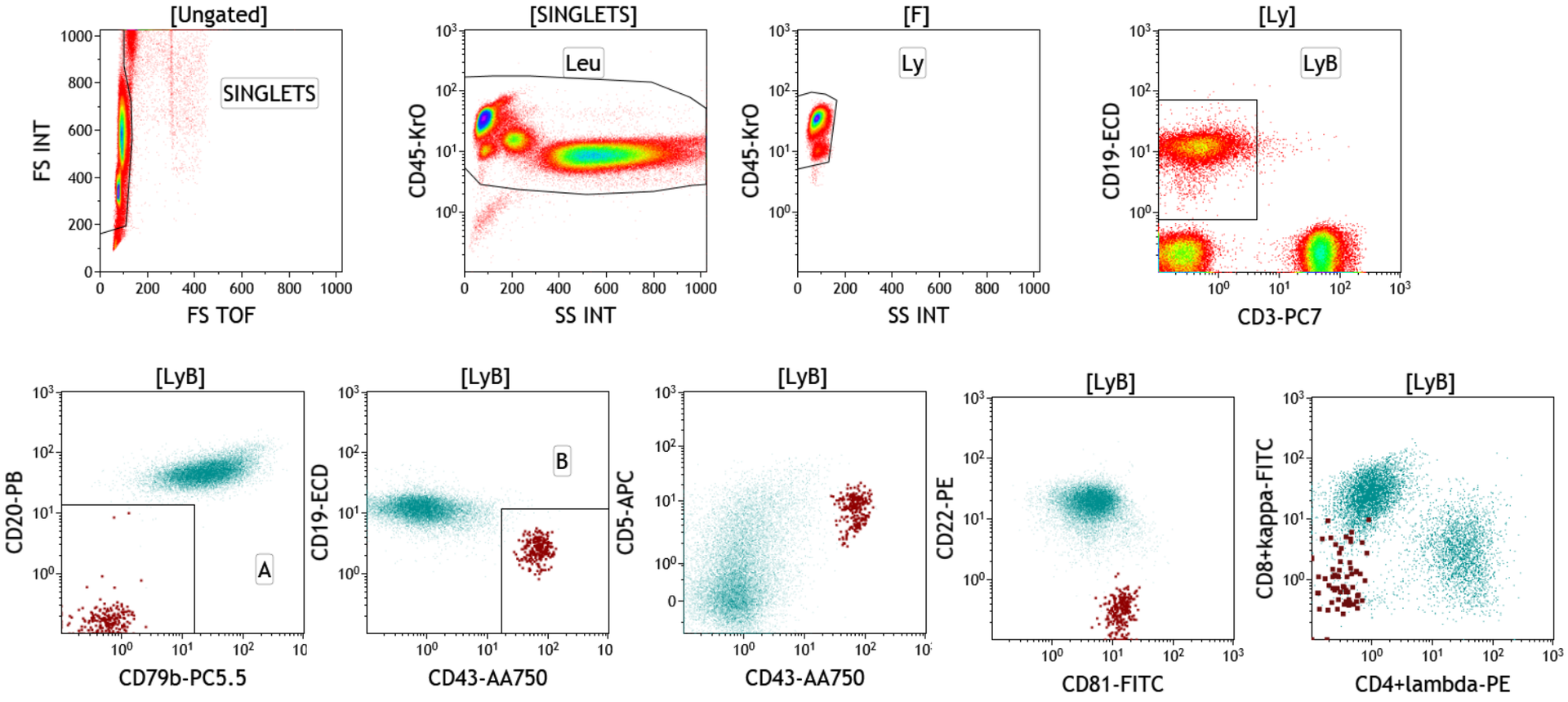
AC Rawstron[1], C Fazi[2], A Agathangelidis[2], N Villamor[3], R Letestu[4], J Nomdedeu[5], C Palacio[6], O Stehlikova[7], K-A Kreuzer[8], S Liptrot[9], D O'Brien[9], RM de Tute[1], I Marinov[10], M Hauwel[11], M Spacek[12], J Dobber[13], AP Kater[13], P Gambell[14], A Soosapilla[15], G Lozanski[16], G Brachtl[17,18], K Lin[19], J Boysen[20], C Hanson[20], JL Jorgensen[21], M Stetler-Stevenson[22], C Yuan[22], HE Broome[23], L Rassenti[23], F Craig[24], J Delgado[3], C Moreno[5], F Bosch[6], A Egle[17], M Doubek[7], S Pospisilova[7], S Mulligan[25], D Westerman[14], CM Sanders[26], R Emerson[26], HS Robins[26], I Kirsch[26], T Shanafelt[20], A Pettitt[19], TJ Kipps[23], WG Wierda[21], F Cymbalista[4], M Hallek[8], P Hillmen[27], E Montserrat[3], and P Ghia[2,28] on behalf of ERIC (European Research Initiative on CLL)

tubo	FITC	PE	PerCP-Cy5.5	PECy7	APC	APCH7	V500	V450
Tubo 1	**CD81**	**CD79b**	CD22	**CD19**	**CD43**	**CD20**	CD3	**CD5**

LOD: 0.001%

Vall d'Hebron

LLC – EMR en sp +1ª final tratamiento (FCR)



MMR: 0.058%

Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF700	AAF750	PaB	KrO
LLC-MMR1	Kappa + CD8	Lambda + CD4	CD19	CD56		CD5		CD3	CD20	CD45
LLC-MMR2	CD81	CD22	CD19	CD79b	CD3	CD5		CD43	CD20	CD45

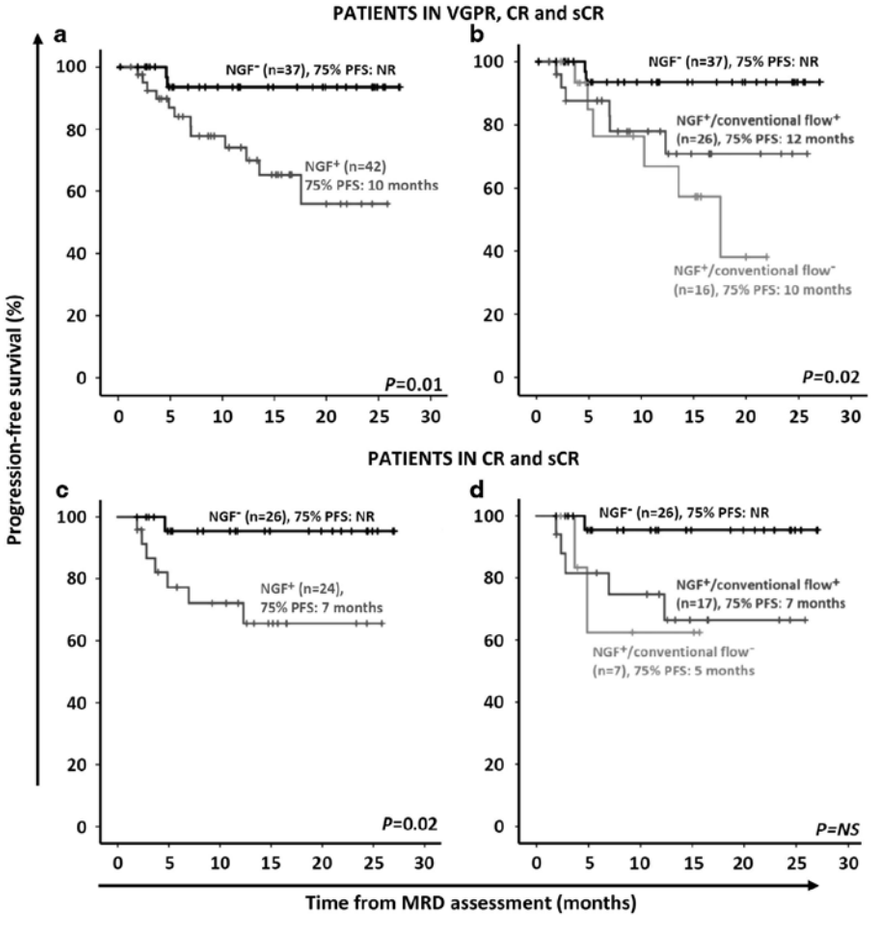
Vall d'Hebron

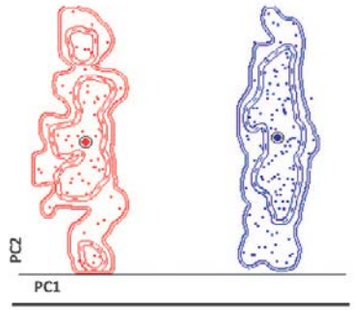
EMR - Mieloma

IMWG MRD criteria (requires a complete response as defined below)	
Sustained MRD-negative	MRD negativity in the marrow (NGF or NGS, or both) and by imaging as defined below, confirmed minimum of 1 year apart. Subsequent evaluations can be used to further specify the duration of negativity (eg, MRD-negative at 5 years)†
Flow MRD-negative	Absence of phenotypically aberrant clonal plasma cells by NGF‡ on bone marrow aspirates using the EuroFlow standard operation procedure for MRD detection in multiple myeloma (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Sequencing MRD-negative	Absence of clonal plasma cells by NGS on bone marrow aspirate in which presence of a clone is defined as less than two identical sequencing reads obtained after DNA sequencing of bone marrow aspirates using the LymphoSIGHT platform (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells§ or higher
Imaging-positive MRD-negative	MRD negativity as defined by NGF or NGS plus disappearance of every area of increased tracer uptake found at baseline or a preceding PET/CT or decrease to less mediastinal blood pool SUV or decrease to less than that of surrounding normal tissue¶

Kumar S et al. IMWG consensus criteria. Lancet Oncol 2016

EMR (NGF) - Mieloma





Parameter	% of contribution
CD56	28%
CD19	19%
CD45	17%
CD81	16%
CD138	6%
CD27	4%

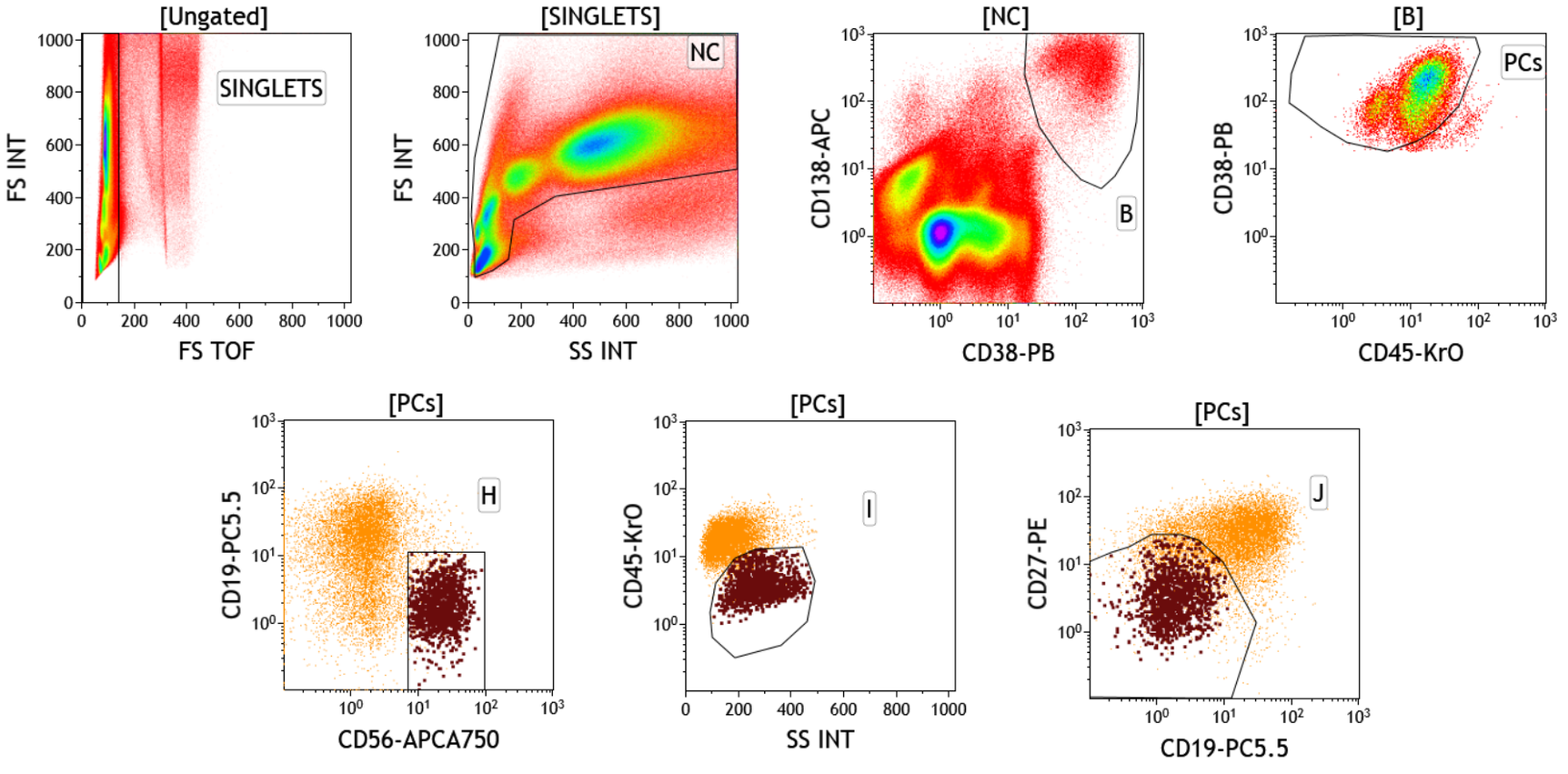
Flores-Montero J, Leukemia 2017

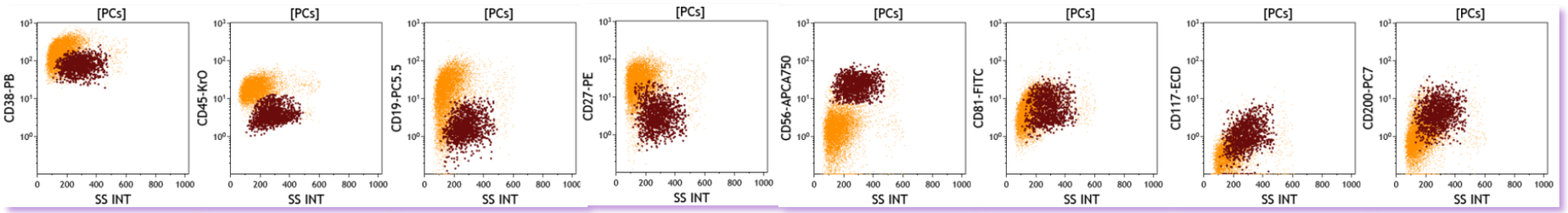
Panel 11. Composition of MM-MRD panel and technical information on reagents*

Tube	BV421	BV510	FITC	PE	PerCPCy5.5	PECy7	APC	APCC750
1	CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81
2	CD138	CD27	CD38	CD56	CD45	CD19	Cylgκ	Cylgλ

Vall d'Hebron

MM en RC – EMR ciclo 15 (KRd)

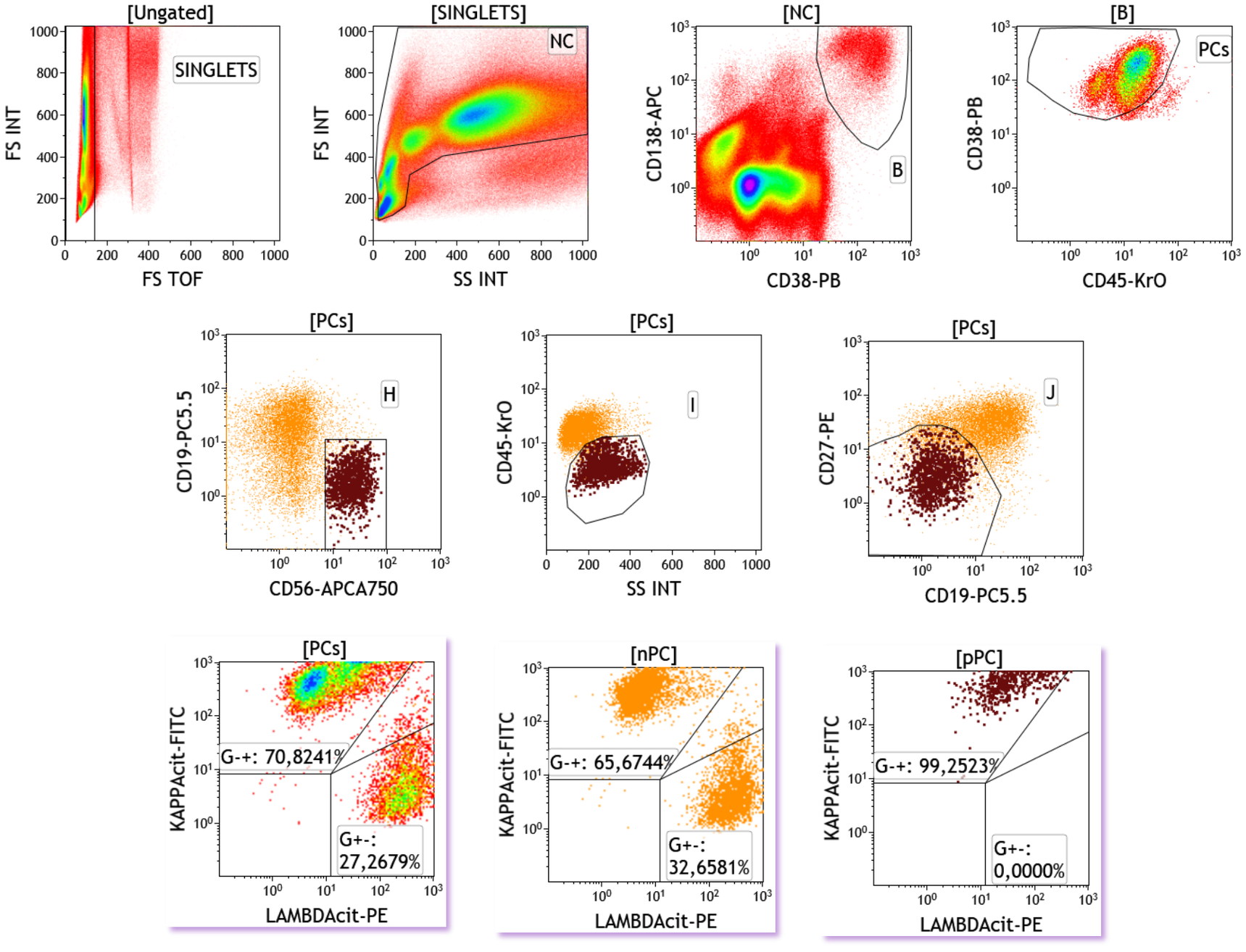




MMR: 0.07%

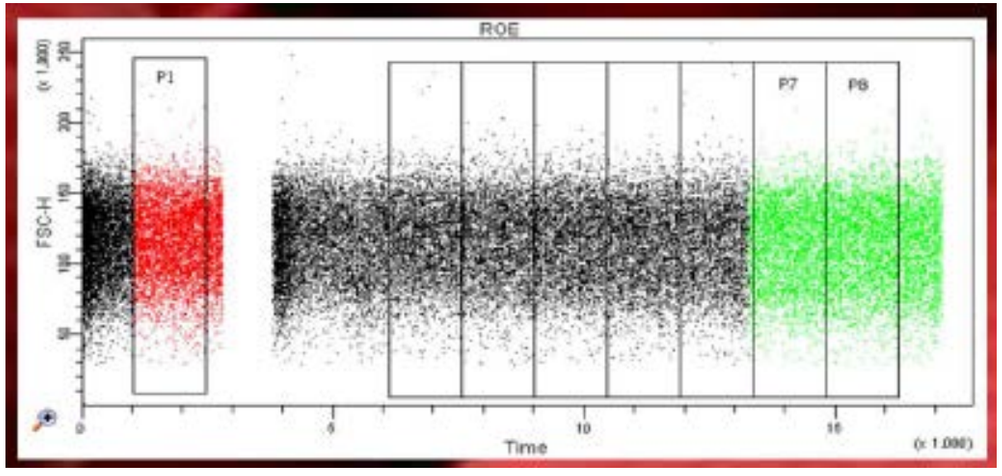
Tub	FITC	PE	ECD	PC5.5	PC7	APC	AAF750	PaB	KrO
MM-1	CD81	CD27	CD117	CD19	CD200	CD138	CD56	CD38	CD45
MM-2	Cit-kappa	Cit-lambda	CD20	CD19		CD138	CD56	CD38	CD45

MM en RC – EMR ciclo 15 (KRd)

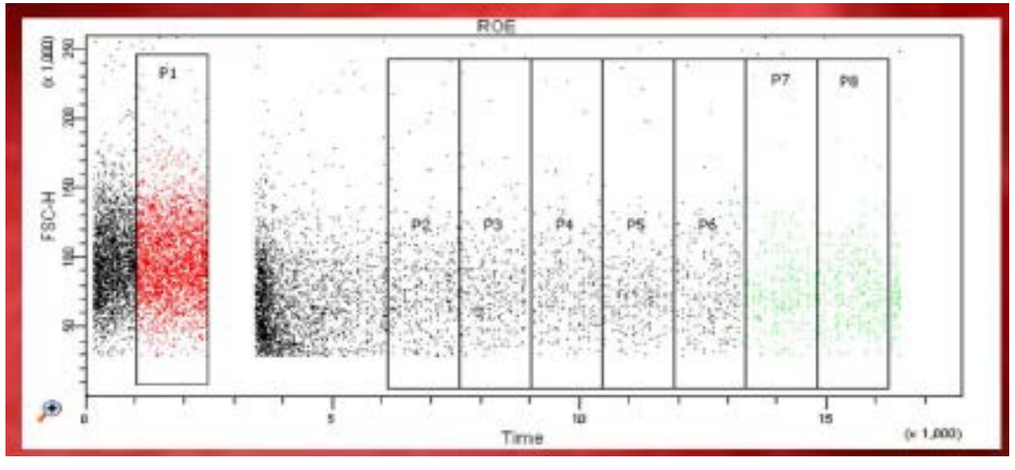


Vall d'Hebron

Resistencia osmótica eritrocitaria (ROE)



ROE normal



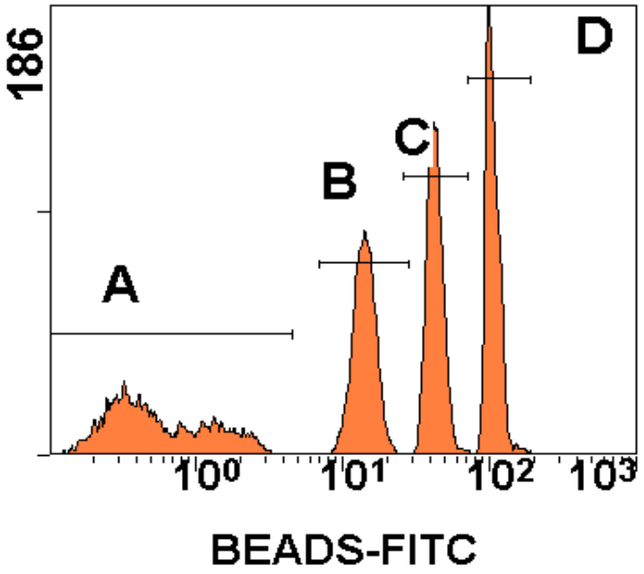
ROE aumentada

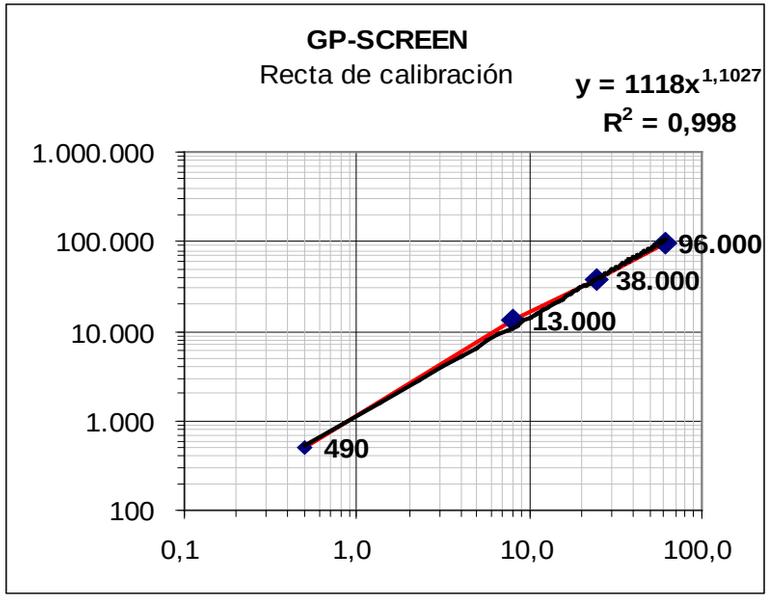
Tiempo

Citometría cuantitativa

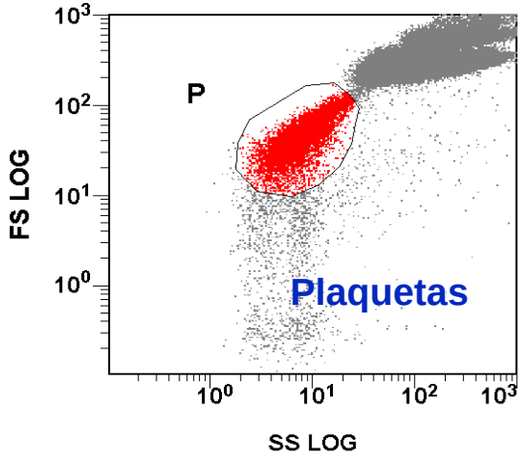
Microesferas marcadas y calibradas con diferentes densidades conocidas de Ag:

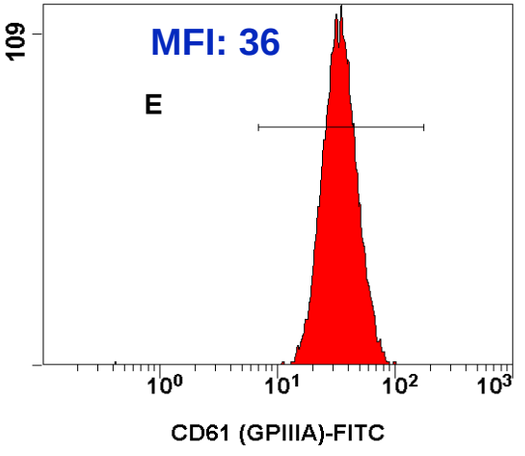
- ✓ Microesferas A: 490 moléculas
- ✓ Microesferas B: 13000 moléculas
- ✓ Microesferas C: 38000 moléculas
- ✓ Microesferas D: 96000 moléculas

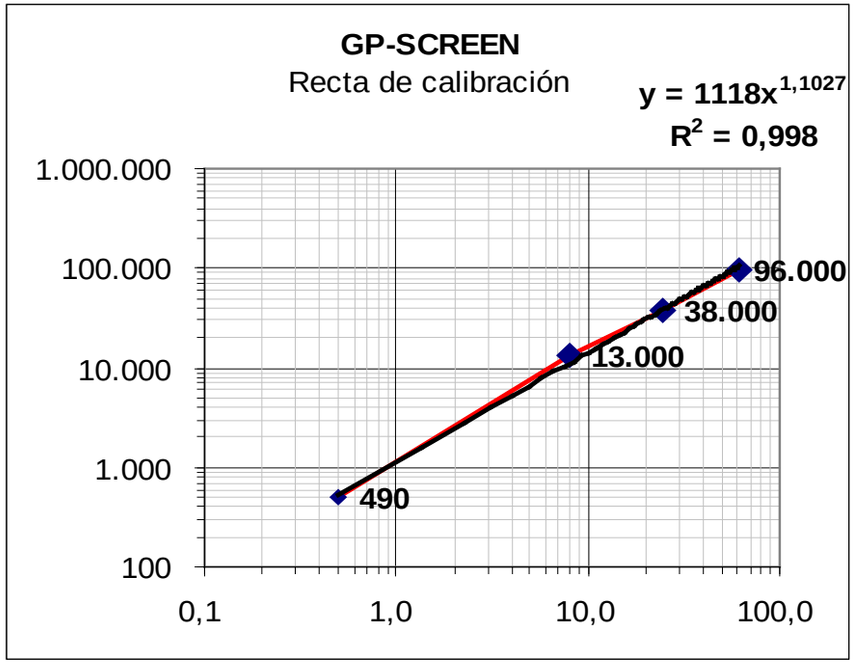




Defectos de Gp de membrana plaquetar





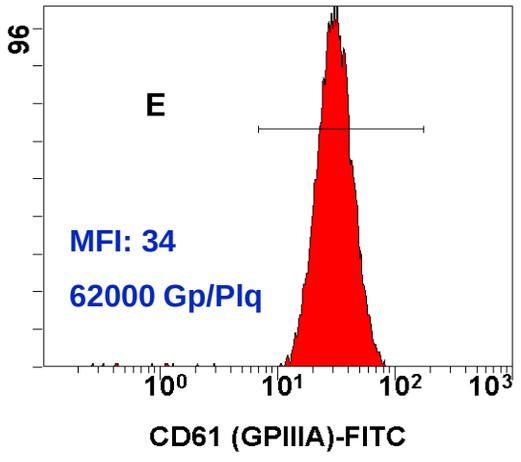


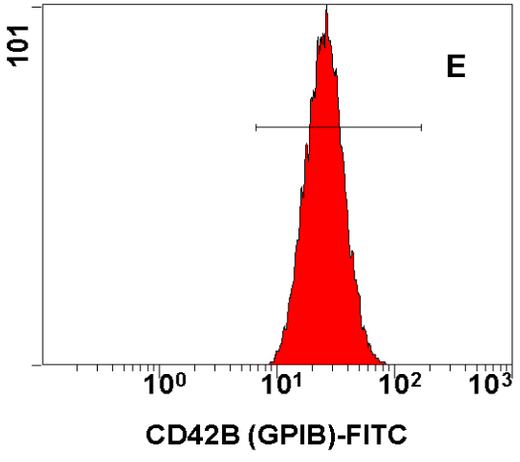
MFI = 36

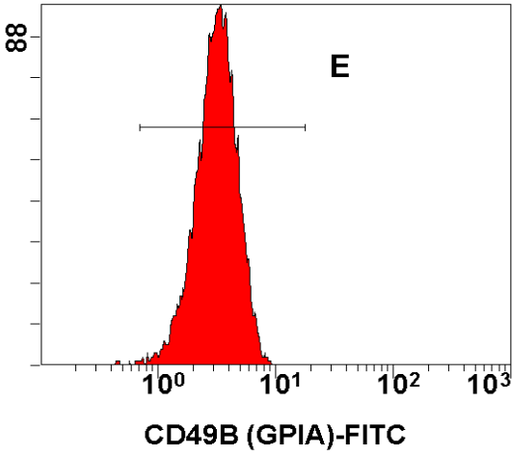
96000 GpIIIa / plaqueta

Defectos de Gp de membrana plaquetar

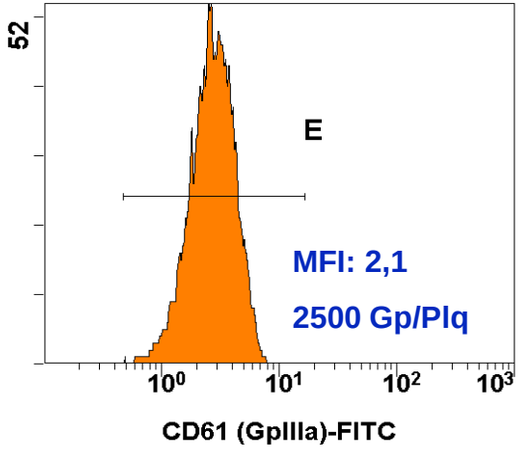
Normal

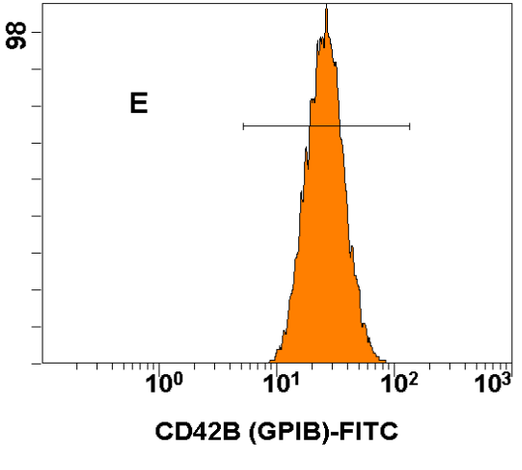


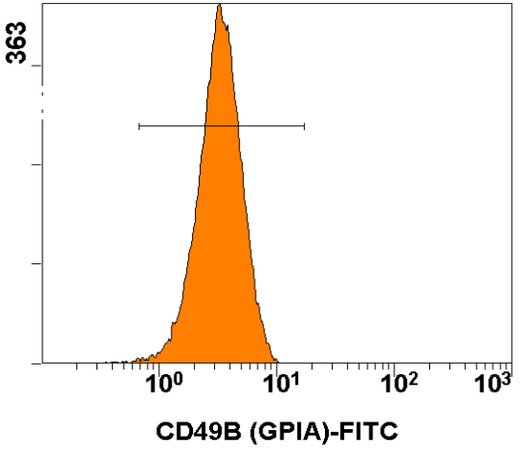




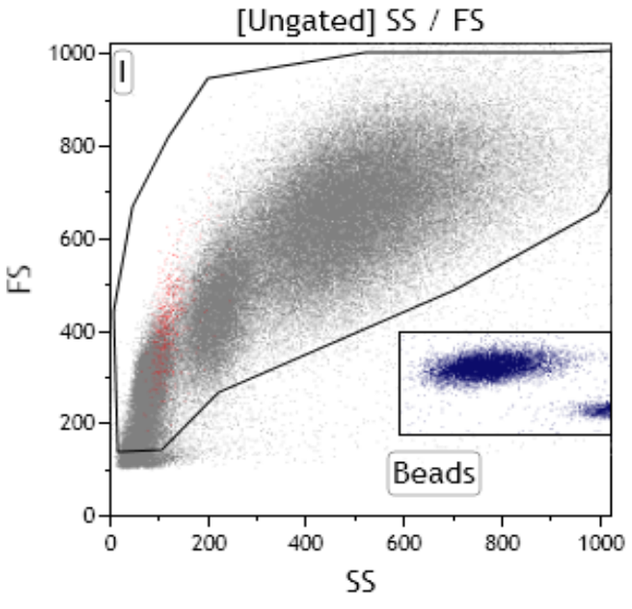
Tromboastenia de Glanzmann

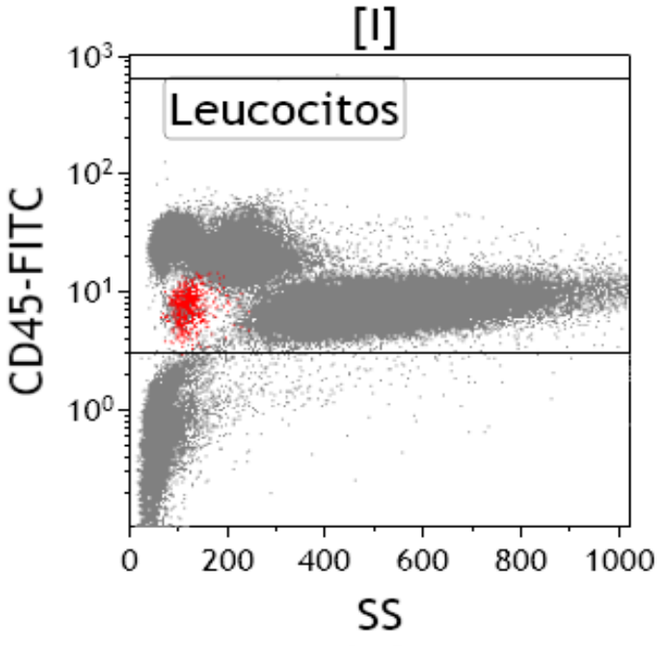


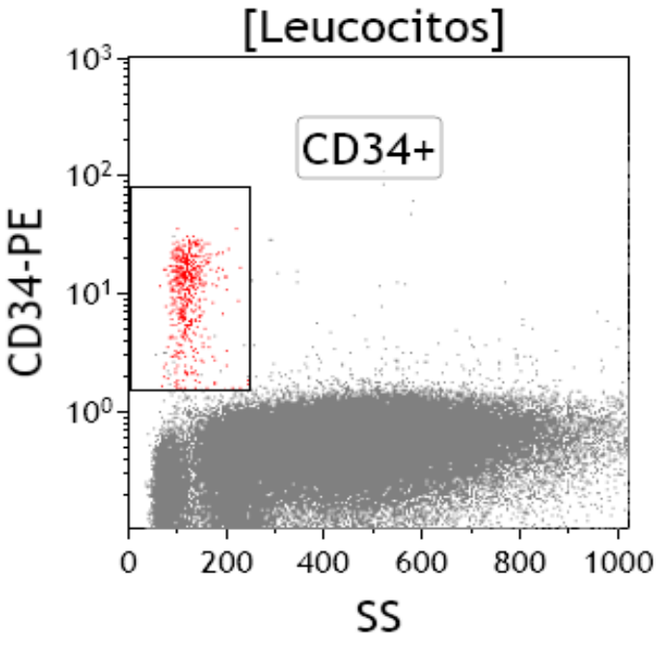


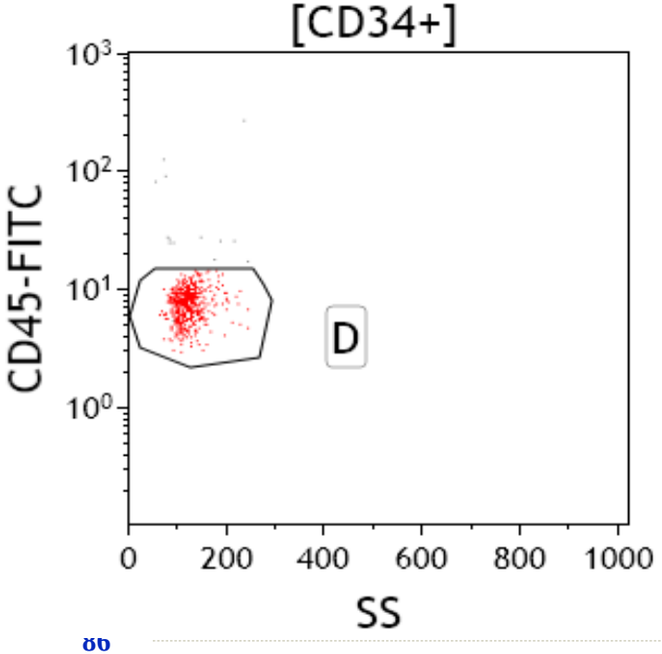


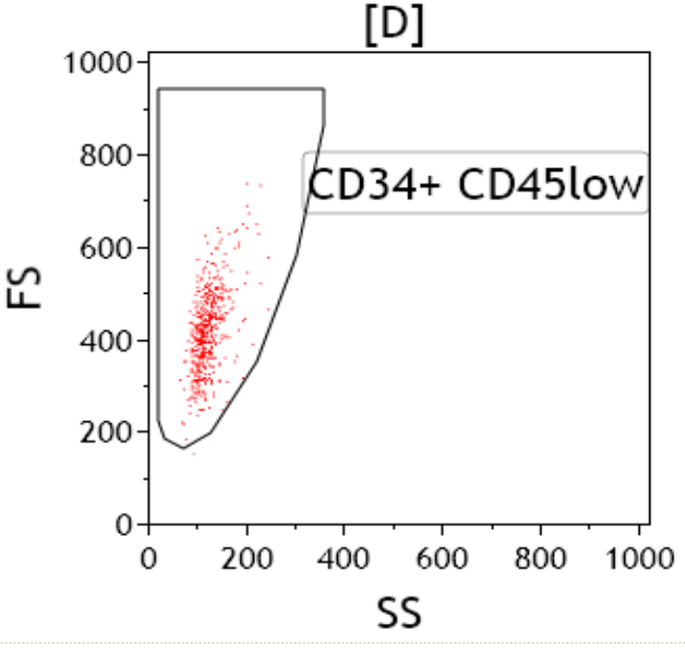
Cuantificación de progenitores CD34+











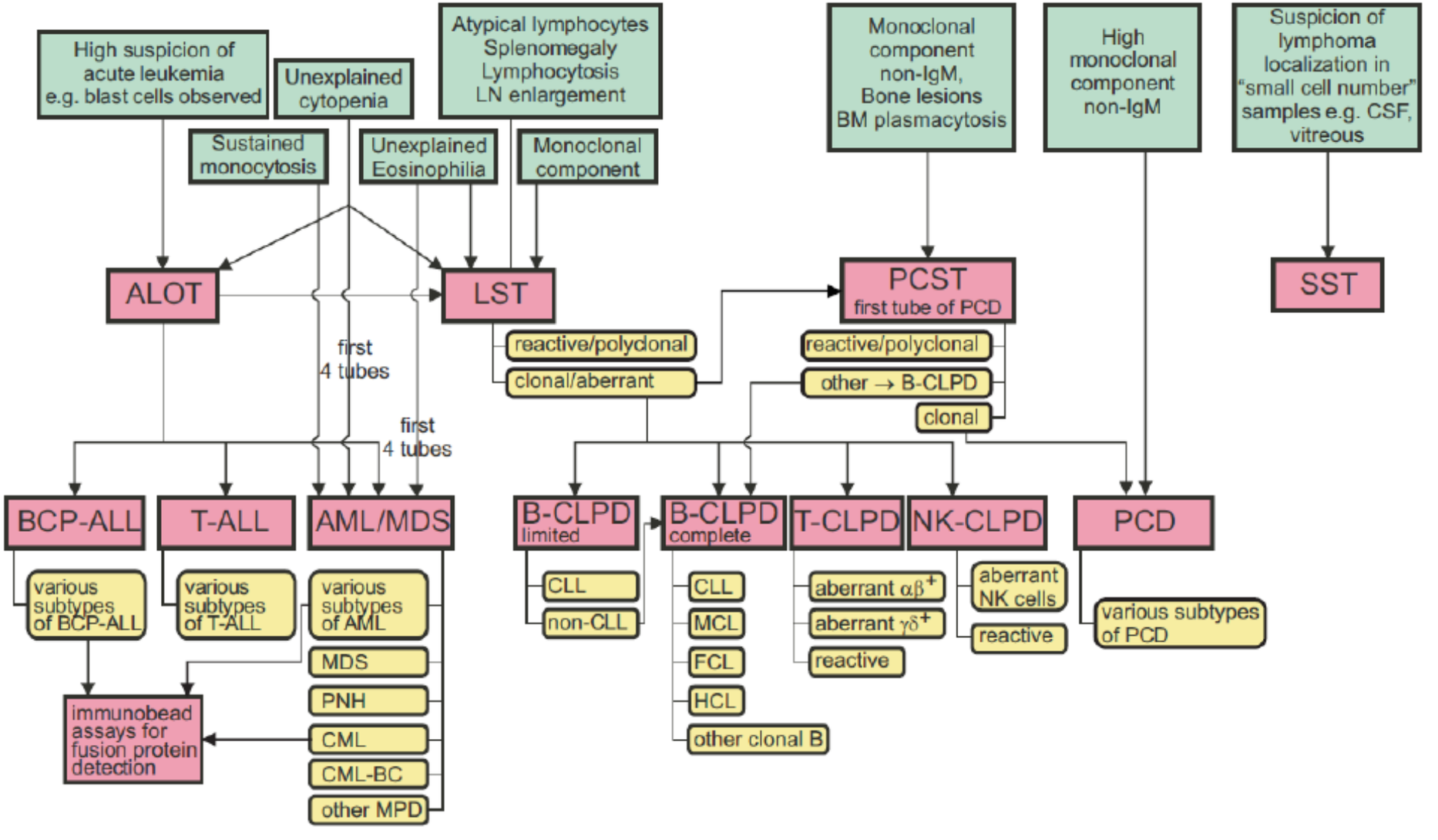
7-AAD+

100000 leucocitos

100 CD34+

Vall d'Hebron

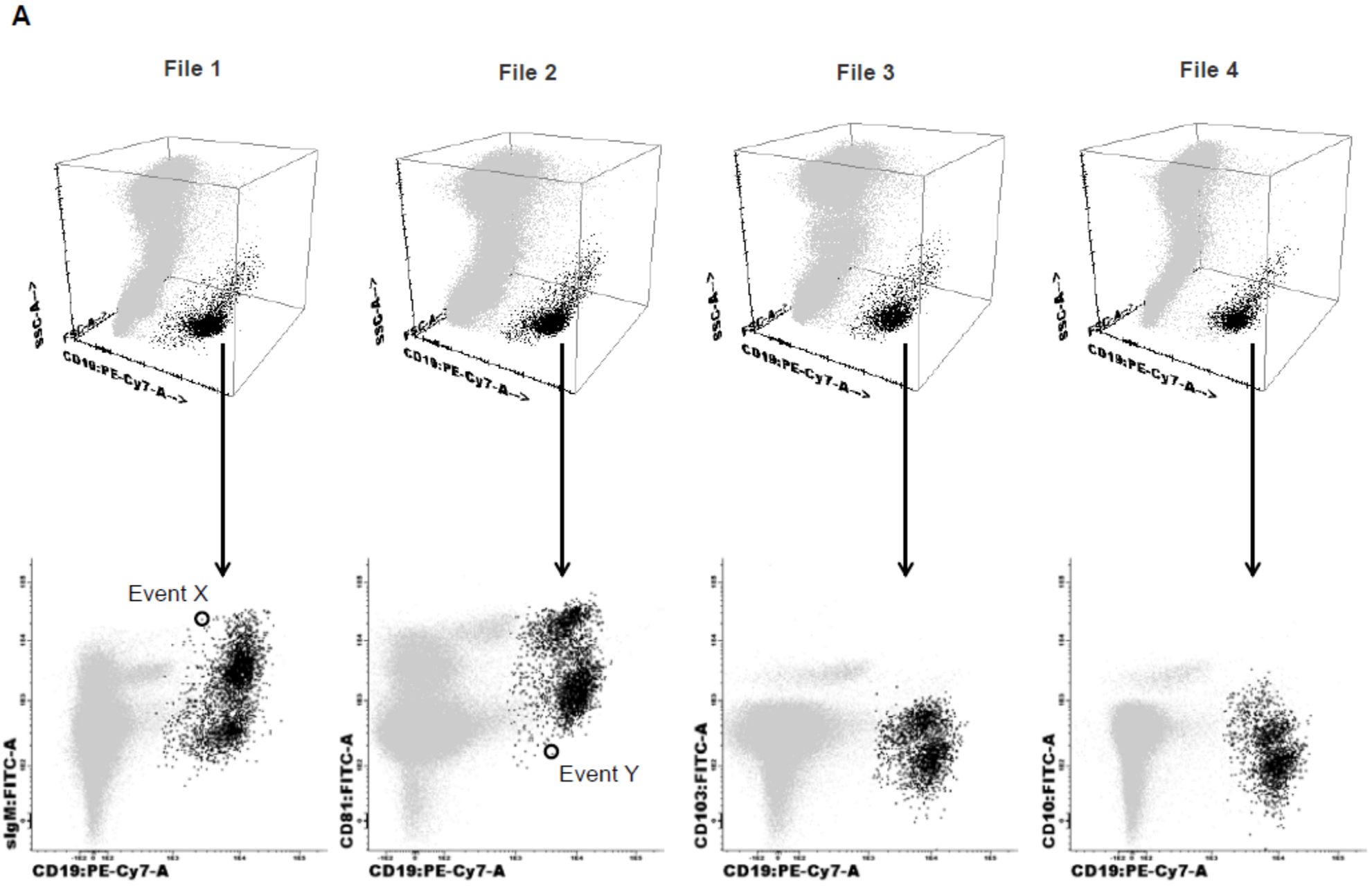
CONSTRUCTION OF EUROFLOW PANELS: MEDICAL INDICATION ORIENTATION/SCREENING & CLASSIFICATION PANELS



Van Dongen et al: EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes.

EuroFlow

Generation of immunophenotypic protein expression profiles (iPEP)



Citometria de flujo policromática

41 parámetros = 1 archivo .fcs

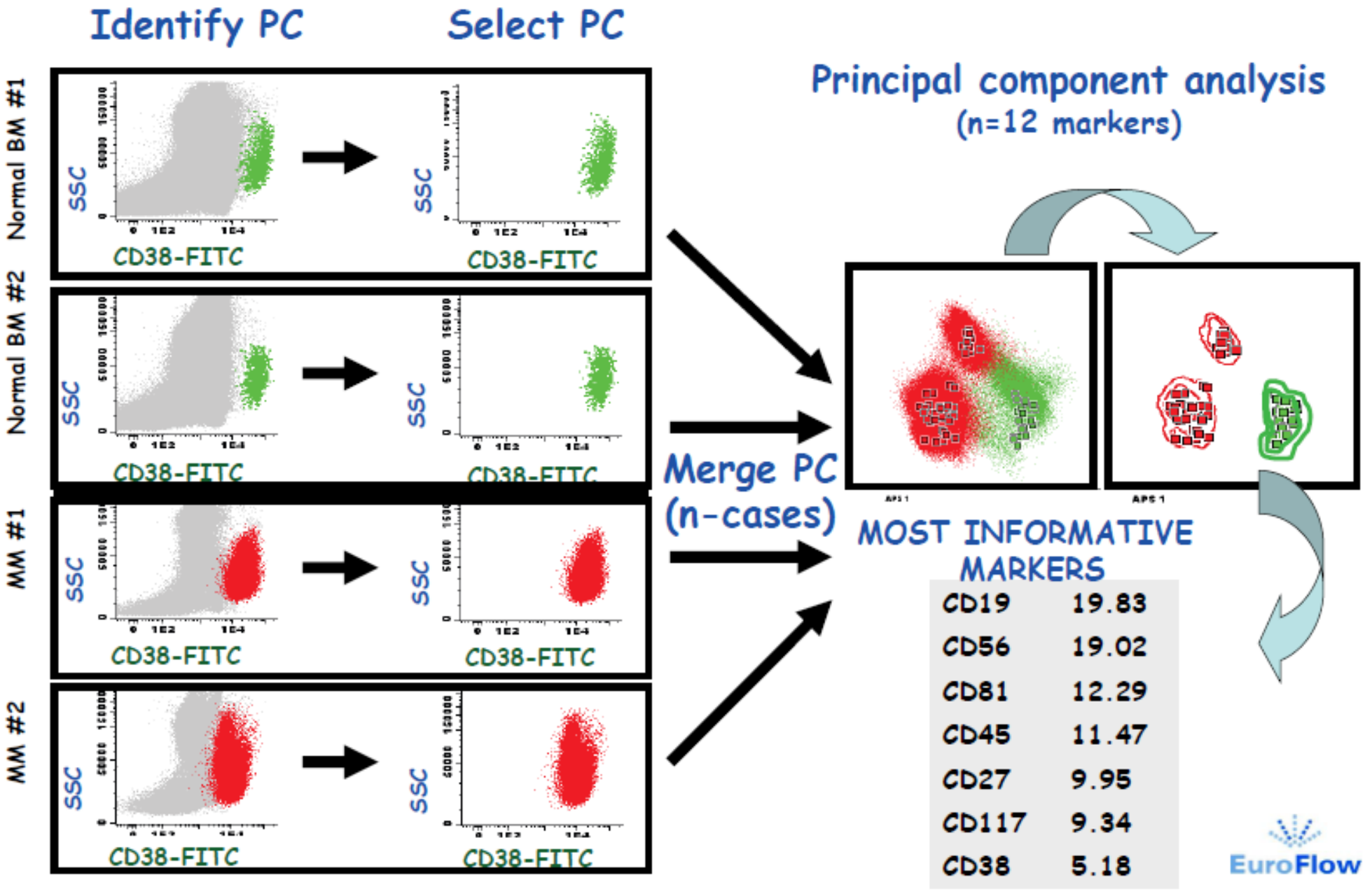
CD45, CD34, CD117, HLA-DR, CyCD3, CyMPO, CyCD79a, CD19, CD7, SmCD3, CD16, CD13, CD11b, CD11c, CD10, CD35, CD64, CD300e, CD14, CD36, CD105, CD33, CD71, CD235a, NuTdT, CD56, CD2, CD7, CD19, CD15, NG2, CD38, CD61, CD203c, CD141, CD123, CD4, CD25, CD42b, CD9, FSC, SSC

820 dot-plots biparamétricos !!!!

Análisis de *clustrers*

PCA (Principal Component Analysis)

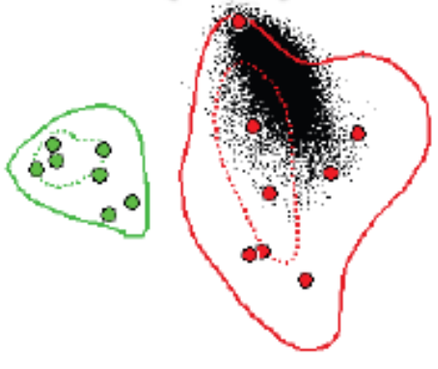
CONSTRUCTION OF EuroFlow MRD PANELS: MM



Automated classification of normal vs aberrant plasma cells in MM

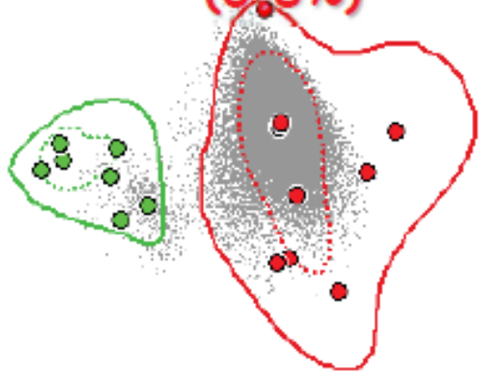
Diagnosis

m-PC (22%)



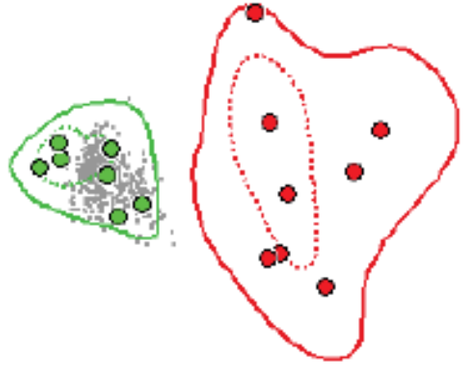
Pre-Transplant

n-PC (0.03%)+m-PC (0.8%)



Post-transplant

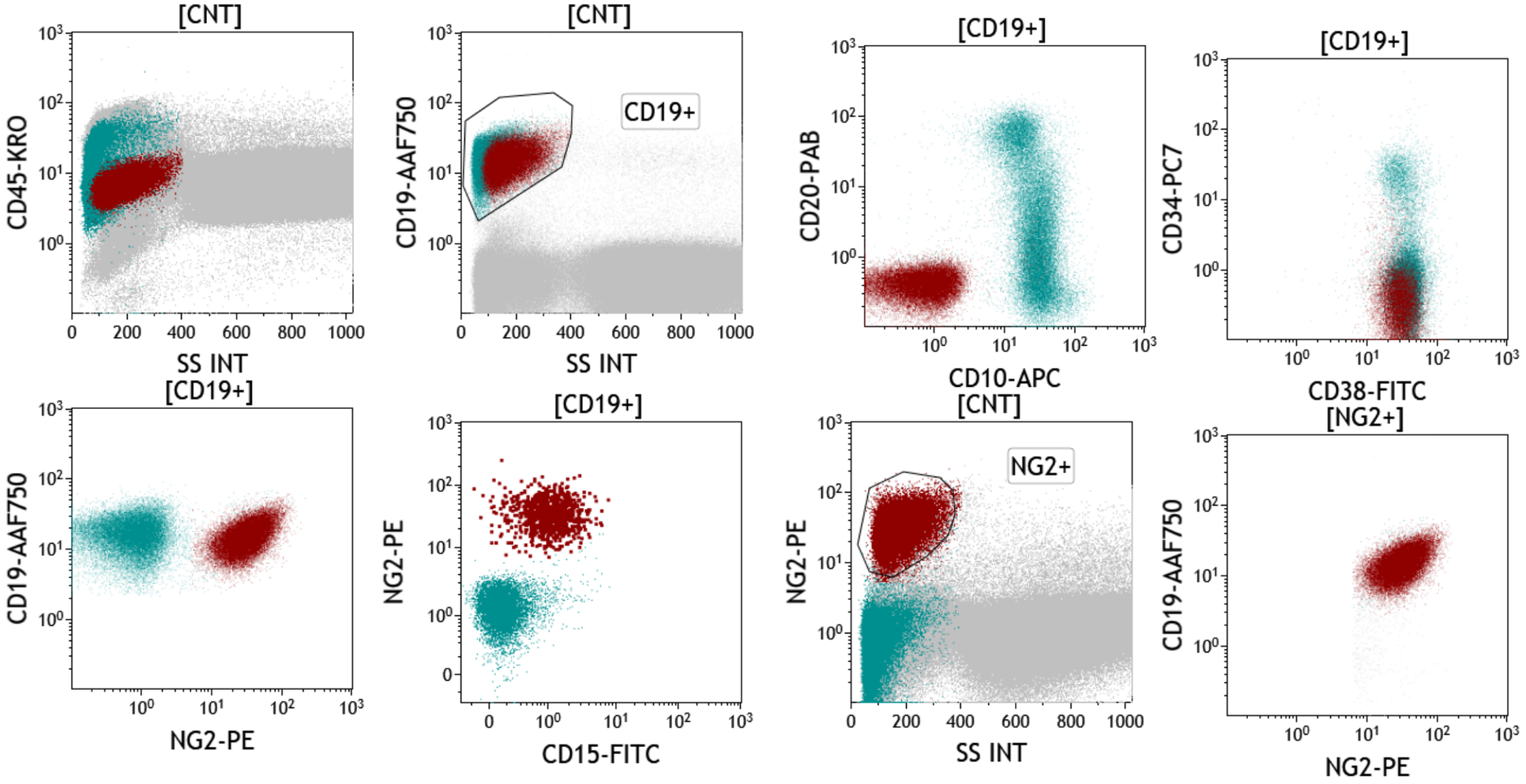
n-PC (0.12%)



Vista Comparativa

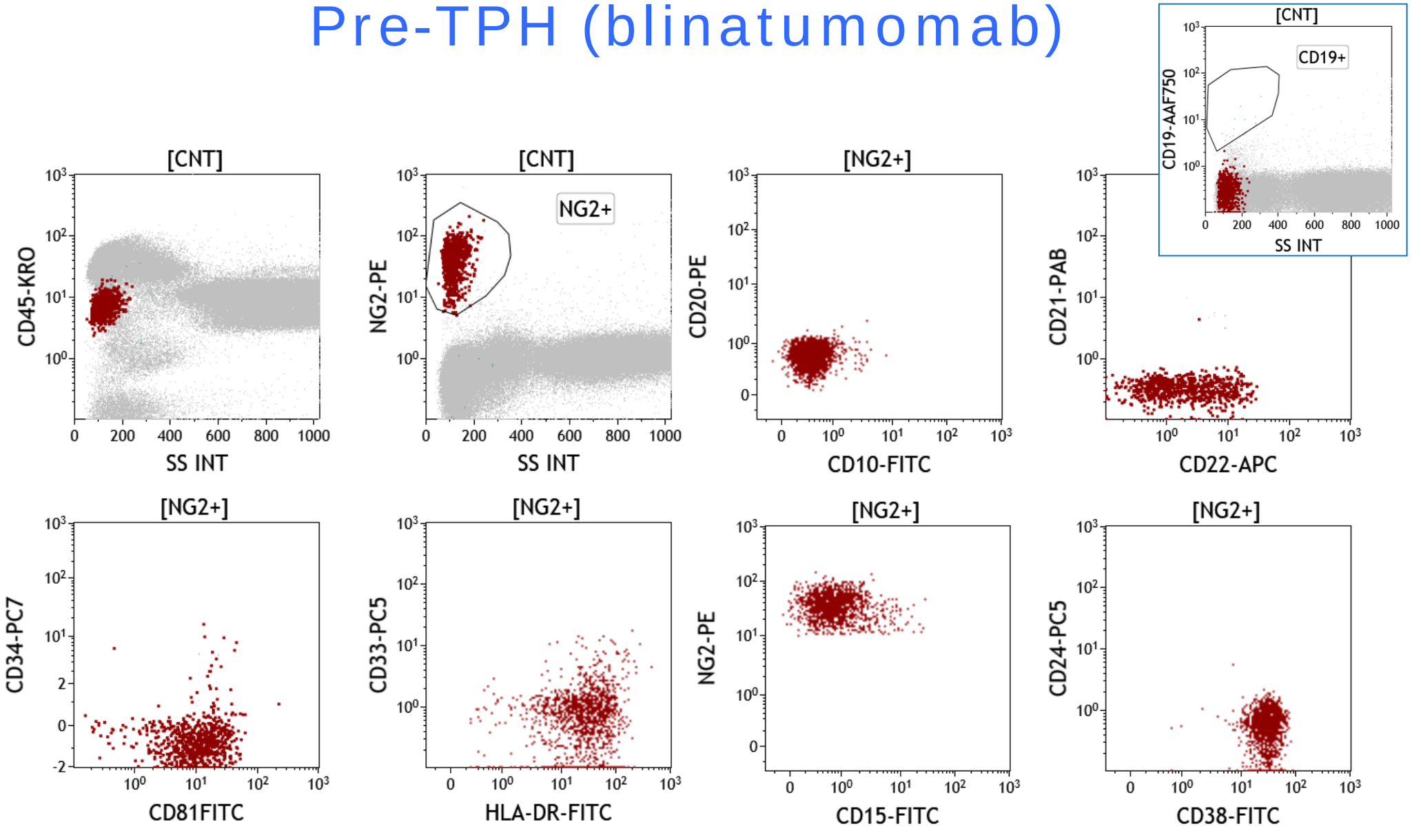
THANK YOU

LLA-B pro-B t(4;11)



EMR (4,36%): ↑SSC, CD19+, CD22+low, CD45+low, CD38+hi, CD34-, CD20-, CD10-, **NG2+hi**

Pre-TPH (blinatumomab)



EMR (0,44%): CD45+low, **CD19-**, CD22+/-, CD38+hi, CD81+hi, CD34-, CD20-, CD21-, CD24-, CD10-, CD15-, CD33- , **NG2+hi**

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LLC – factores pronósticos

- ✓ **Expresión de CD38 (> 20%)**
- ✓ **Expresión de ZAP-70 (> 20%)**

Otros

- ✓ Población T aumentada
- ✓ FMC7+, CD23 ↓, CD5 ↓, cambios en tamaño (transformación)
- ✓ Expresión de CD49d, CD69L
- ✓ Lipoproteína Lipasa A (LPL), ADAM29 (RT-PCR)

LLC – factores pronósticos

Estadios iniciales (Binet A) 65%

- ✓ **25%** **fallecen de causas atribuibles a LLC**
- ✓ **40%** **progresan a estadíos B y C**
- ✓ **50%** **acaban necesitando tratamiento**

LLC – ZAP70

Correspondencia ZAP-70 – mutaciones IgV_H

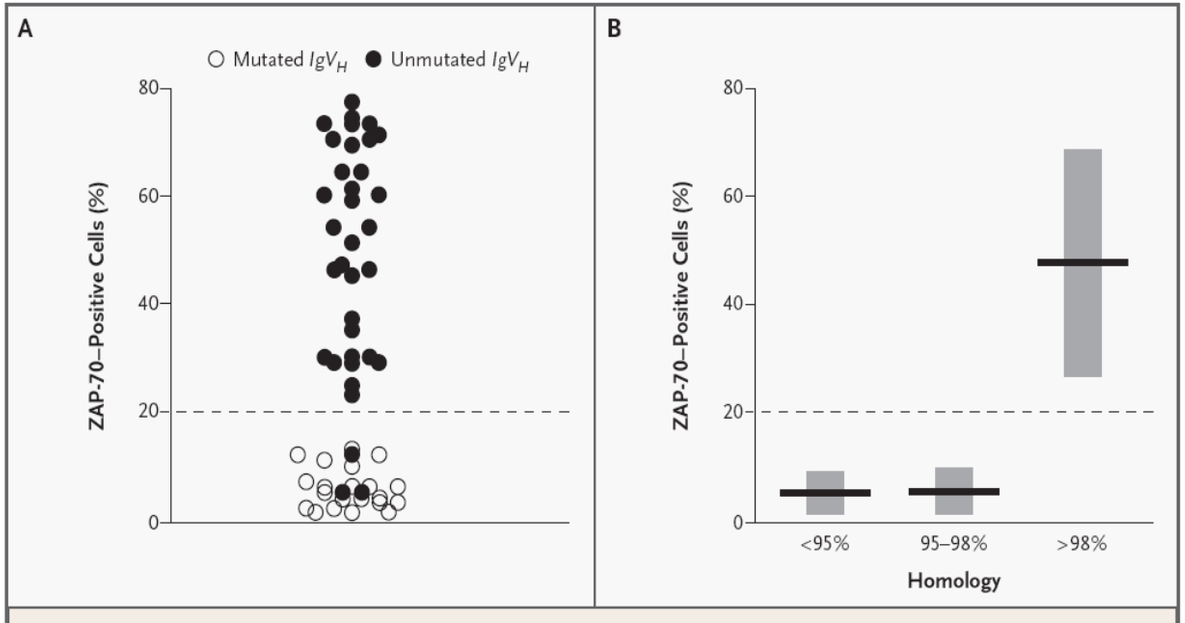

Figure 3. Correlation of the Level of Expression of ZAP-70 and Immunoglobulin Heavy-Chain Variable-Region (IgV_H) Mutational Status (Panel A) and IgV_H Sequence Homology (Panel B).

Panel A shows a scatter plot representing the levels of ZAP-70 expression measured by flow-cytometric analysis and the IgV_H mutational status of the 56 patients analyzed. When the threshold for categorizing ZAP-70 was established at 20 percent, two subgroups were clearly delineated: patients with unmutated IgV_H and a high level of ZAP-70 expression and patients with mutant IgV_H and low levels of ZAP-70 expression. Only three patients without IgV_H mutations had low levels of ZAP-70 expression. Panel B shows the mean (±SD) (horizontal line and box) level of expression of ZAP-70 according to the degree of homology of each IgV_H sequence with the consensus sequence (less than 95 percent, 95 to 98 percent, or more than 98 percent). There were no significant differences in the level of ZAP-70 expression between the subgroup with less than 95 percent sequence homology and the subgroup with 95 to 98 percent sequence homology.

n=56

ZAP-70 -: 100% MuIgV_H

ZAP-70 +: 88% UmIgVH

Crespo et al. N Engl J Med 2003

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Leucemias agudas

Mutaciones de tipo I

FLT3-ITD

FLT3-TKD

KIT

RAS

PTPN11

JAK2

Mutaciones de tipo II

PML-RARA

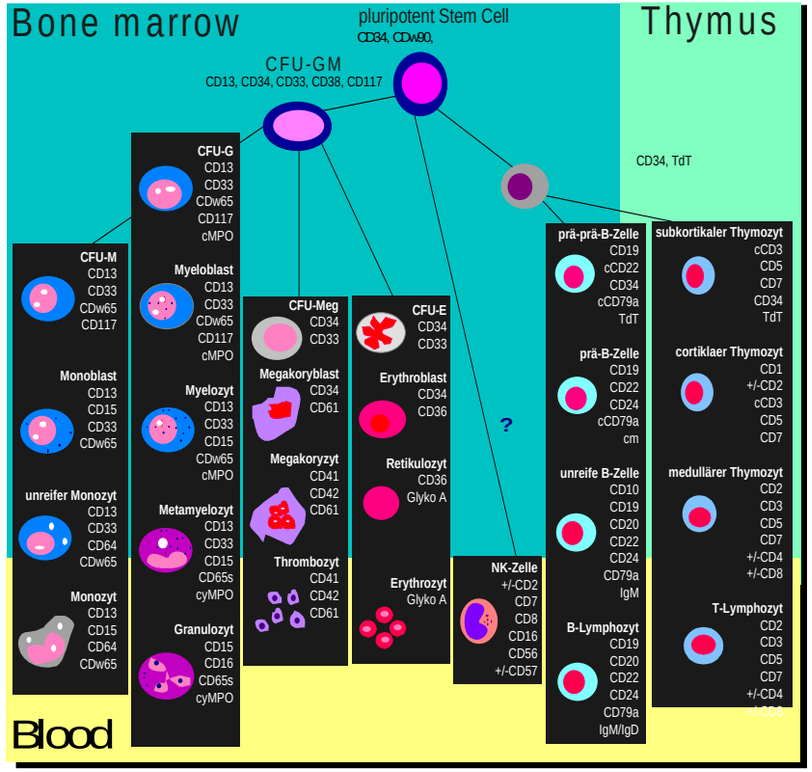
RUNX1-RUNX1T1

CBF-MYH11

Fusiones de MLL

CEBPA

NPM1



Proliferación

Supervivencia

Diferenciación

LEUCEMIA MIELOBLÁSTICA AGUDA

WHO 2008

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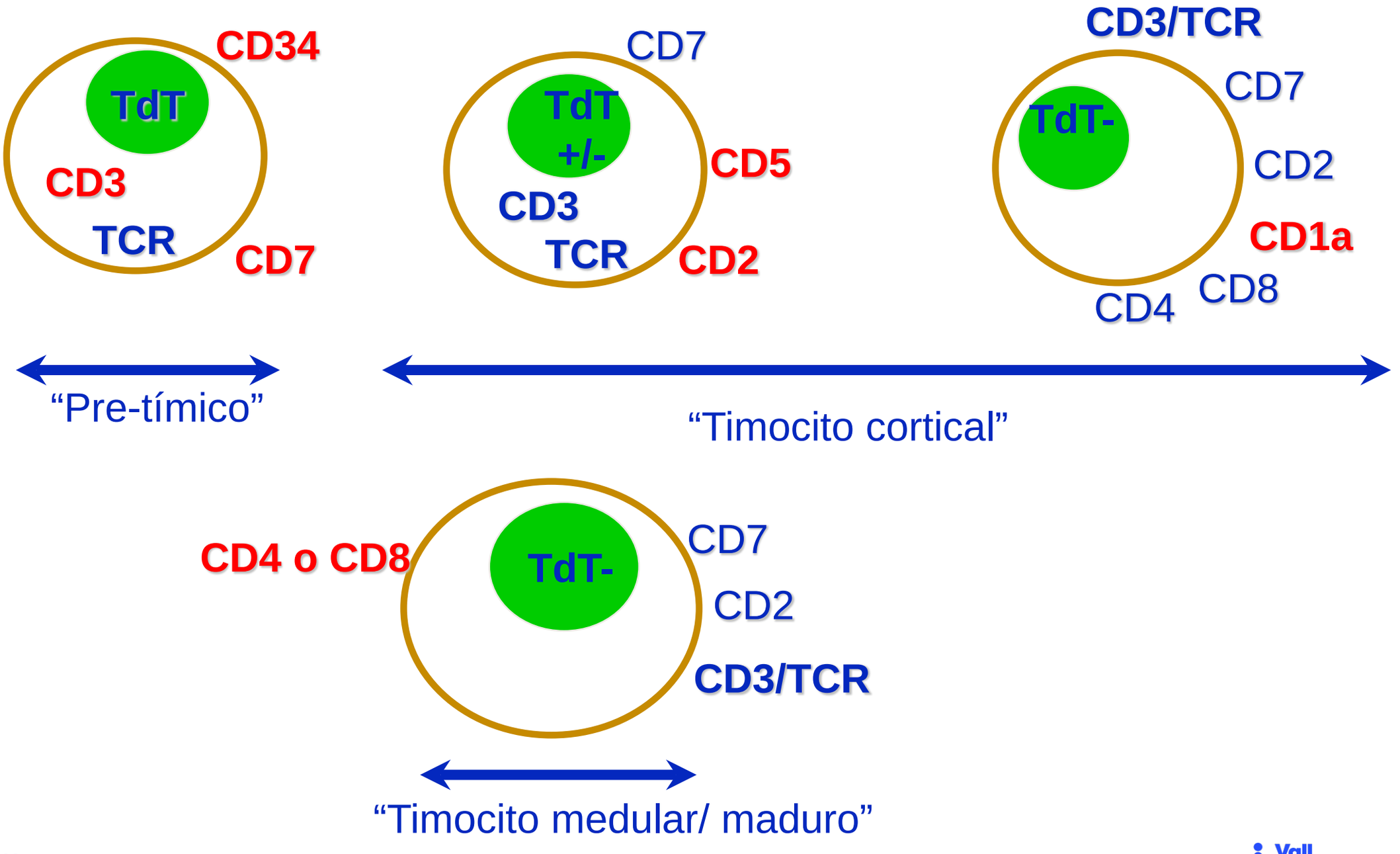
Diferenciación linfoide T



LLA-T (EGIL)

1) **LLA pro-T (TI):** CD3+(cit), CD7+

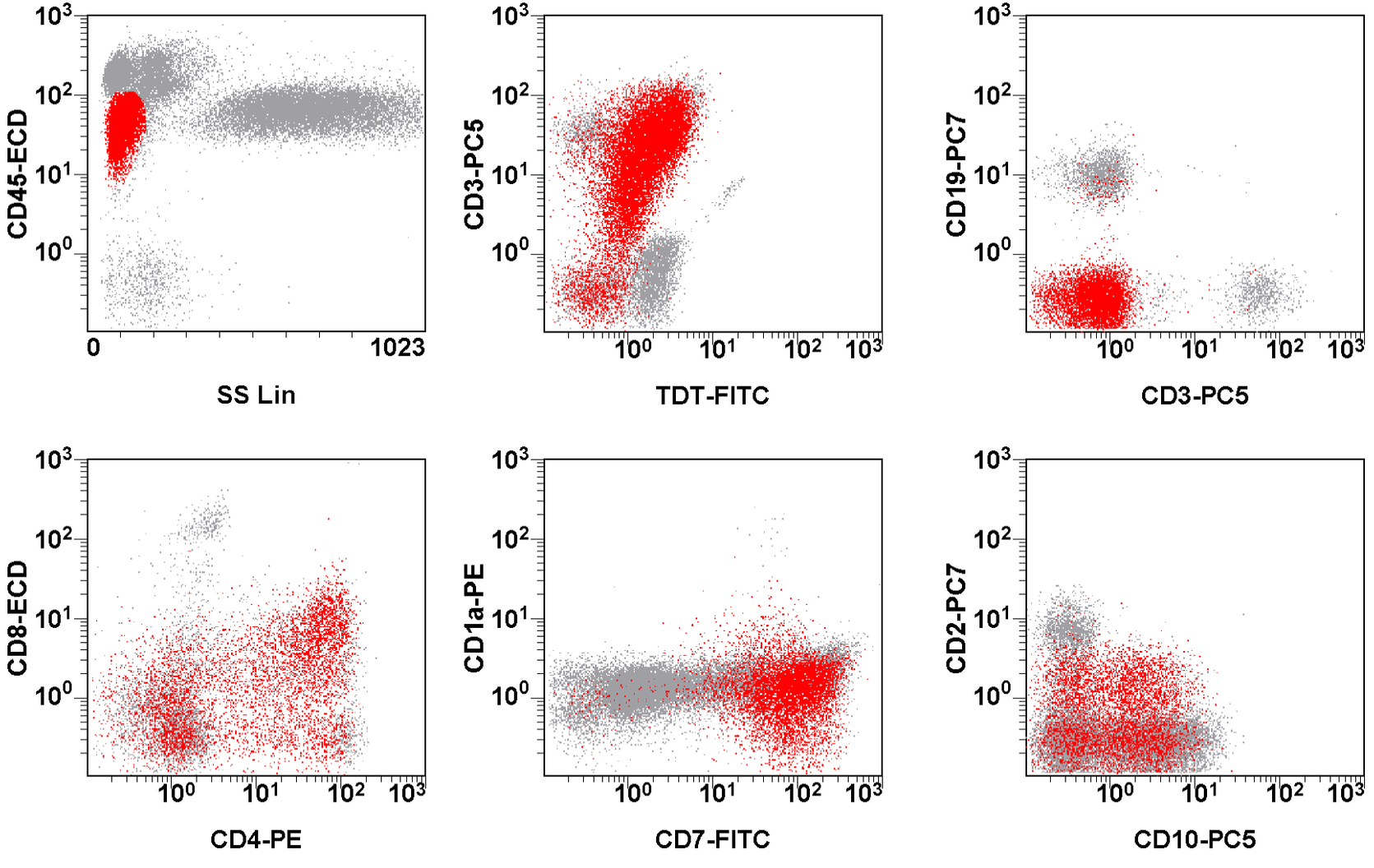
2) **LLA pre-T (TII):** CD3+(cit), CD2+ y/o CD5+ y/o CD8+

3) **LLA-T cortical (TIII):** CD3+ (empieza a expresarse en membrana), CD1a+ . El resto de los marcadores son indiferentes

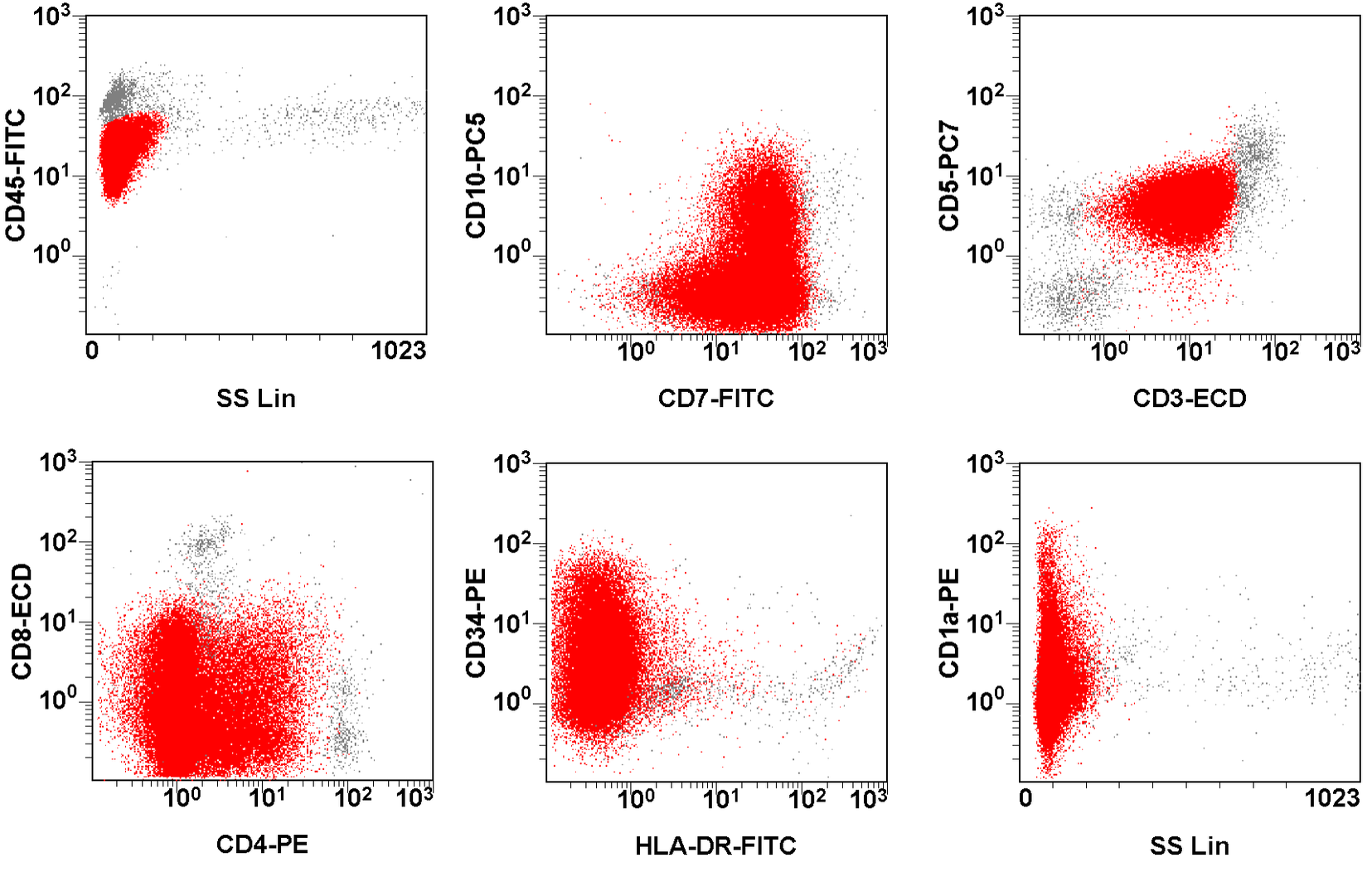
4) **LLA-T medular o madura (TIV):** CD3+ (membrana), CD1a-

Grupo A: TCR $\alpha\beta$ + ; Grupo B: TCR $\gamma\delta$ +

LLA-T ‘pre-T’ - sangre



LLA-T ‘tímica cortical’



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"CURS CITOMETRIA DE FLUX: ASPECTES TEÒRICS I APLICACIONS¨

Aplicació de la citometria de flux al screening diagnòstic i monitorització de malalties immunomediades e immunodeficiències primàries

Dra. Mónica Martínez Gallo

Vall d'Hebron Campus Hospitalari

Vall d'Hebron Barcelona Hospital Campus

27 març 2018

Immunodeficiency. Definition

- **Definition**

Immunodeficiency is the result of a diverse group of abnormalities of the immune system resulting primarily in an increased incidence of infection.

- **Primary Immunodeficiency**

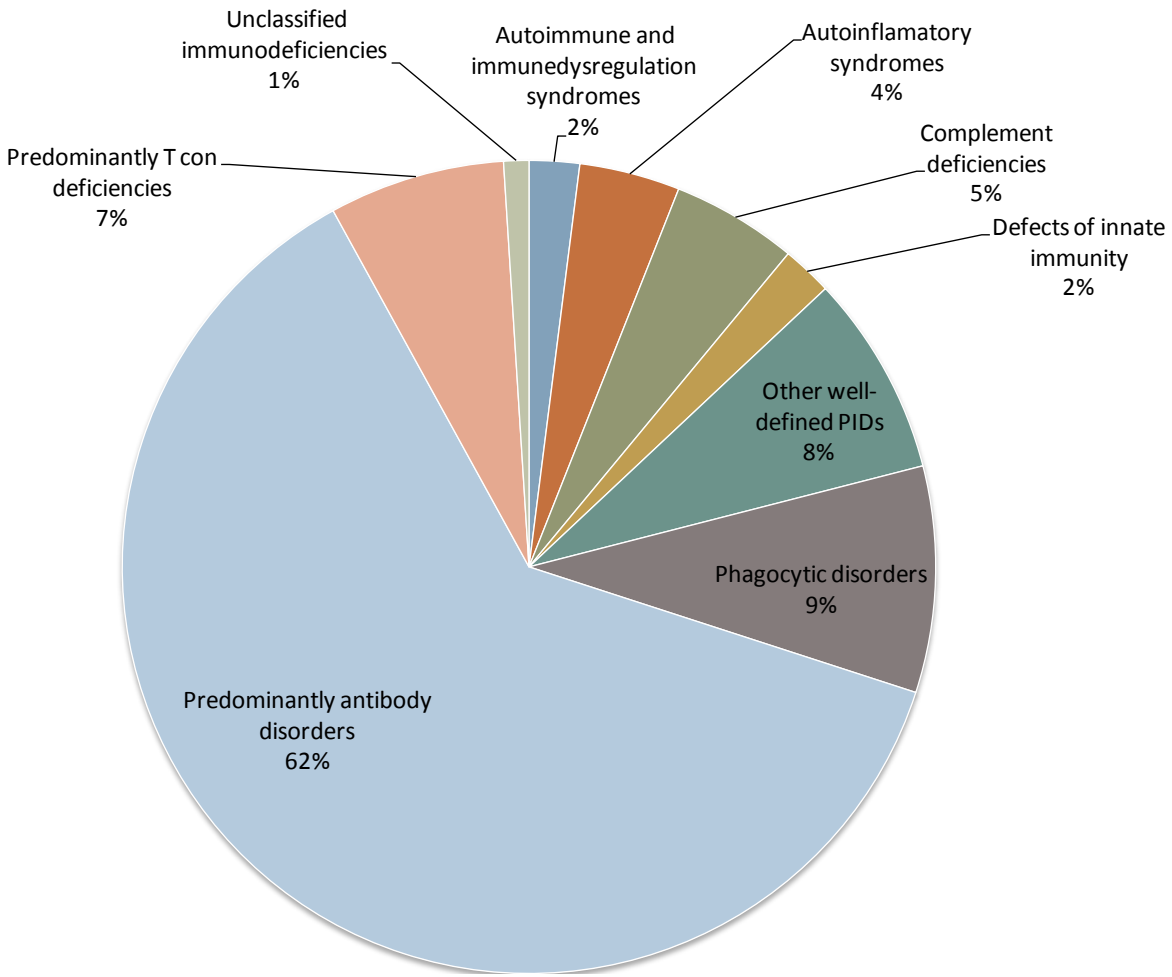
Congenital and hereditary

- **Secondary Immunodeficiency**

Acquired on a transient or permanent basis

- AIDS
- Corticosteroids
- Chemotherapy
- Irradiation
- Malnutrition - Stress - Exhaustion

Distribution of Primary Immunodeficiency Diseases



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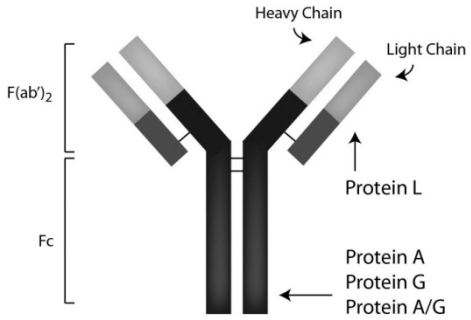
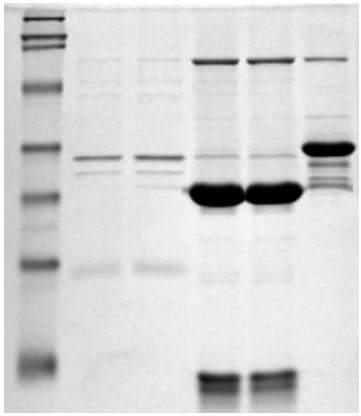
Immunology Diagnostic Laboratory

- Immunochemistry
- Immunoallergy
- Autoimmunity
- Cellular Immunology
- Immunogenetics





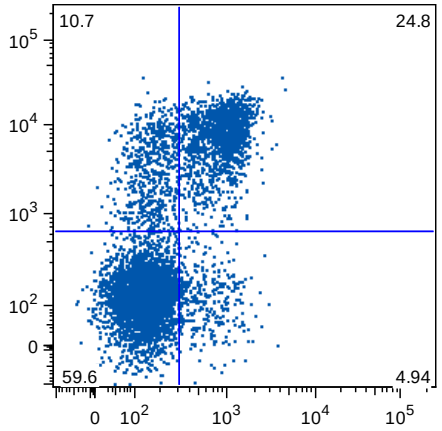


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Table 2 Algorithm to evaluate a suspected cellular immune defect

Screening Evaluation	• CBC with differential • Immunophenotyping ○ T-, B-, NK-cell counts and CD45RA/RO^{+} status
Advanced Testing (*If pertinent, based upon previous testing results or high clinical suspicion*)	• Functional testing ○ DTH testing ○ Mitogen stimulation ○ Cytotoxicity assay • TREC assay • Genetic evaluation • Advanced flow studies ○ i.e., TH17, CD40L, WASp, etc.

Clinic Rev Allerg Immunol (2014) 46:154–168

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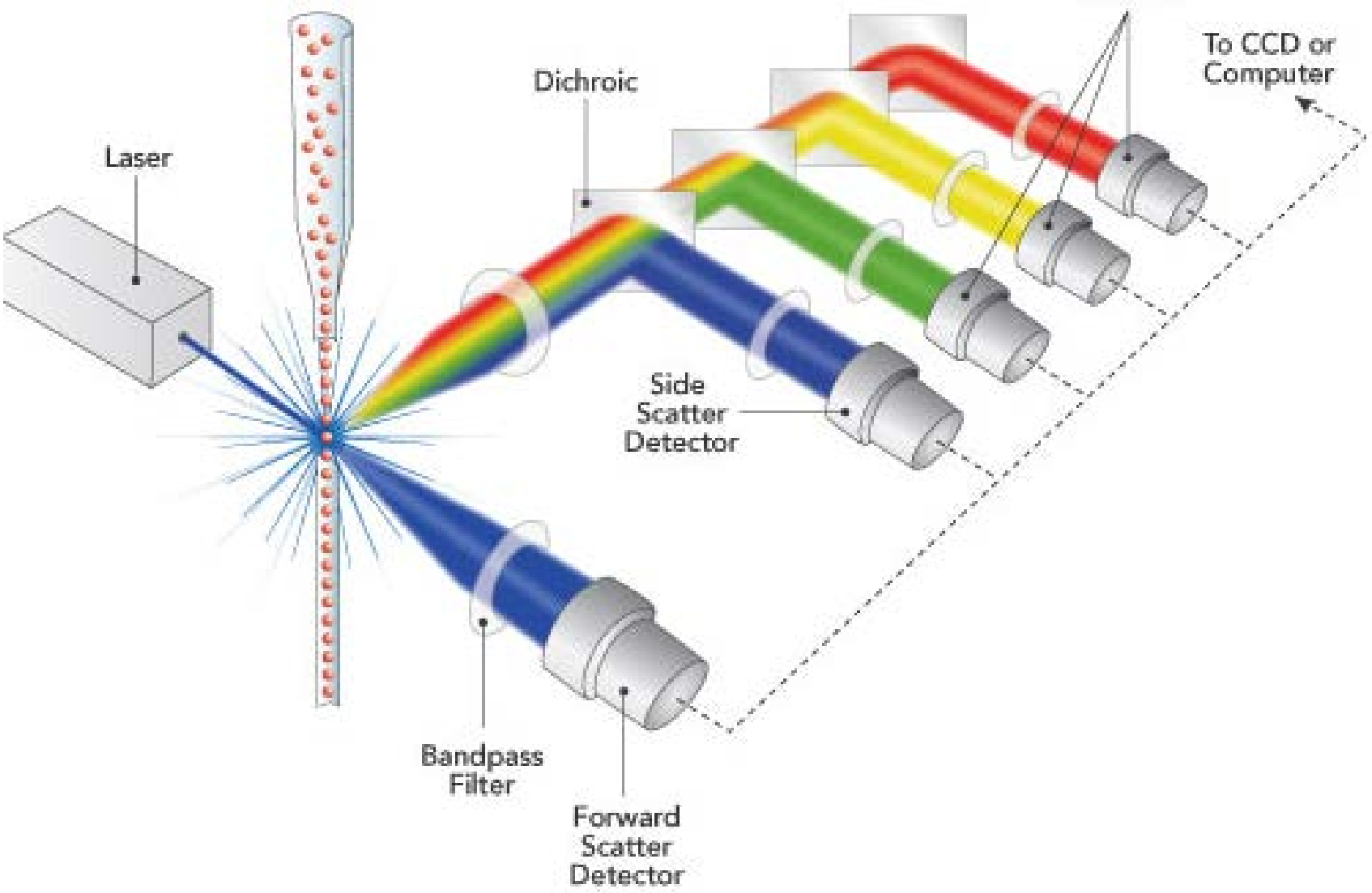
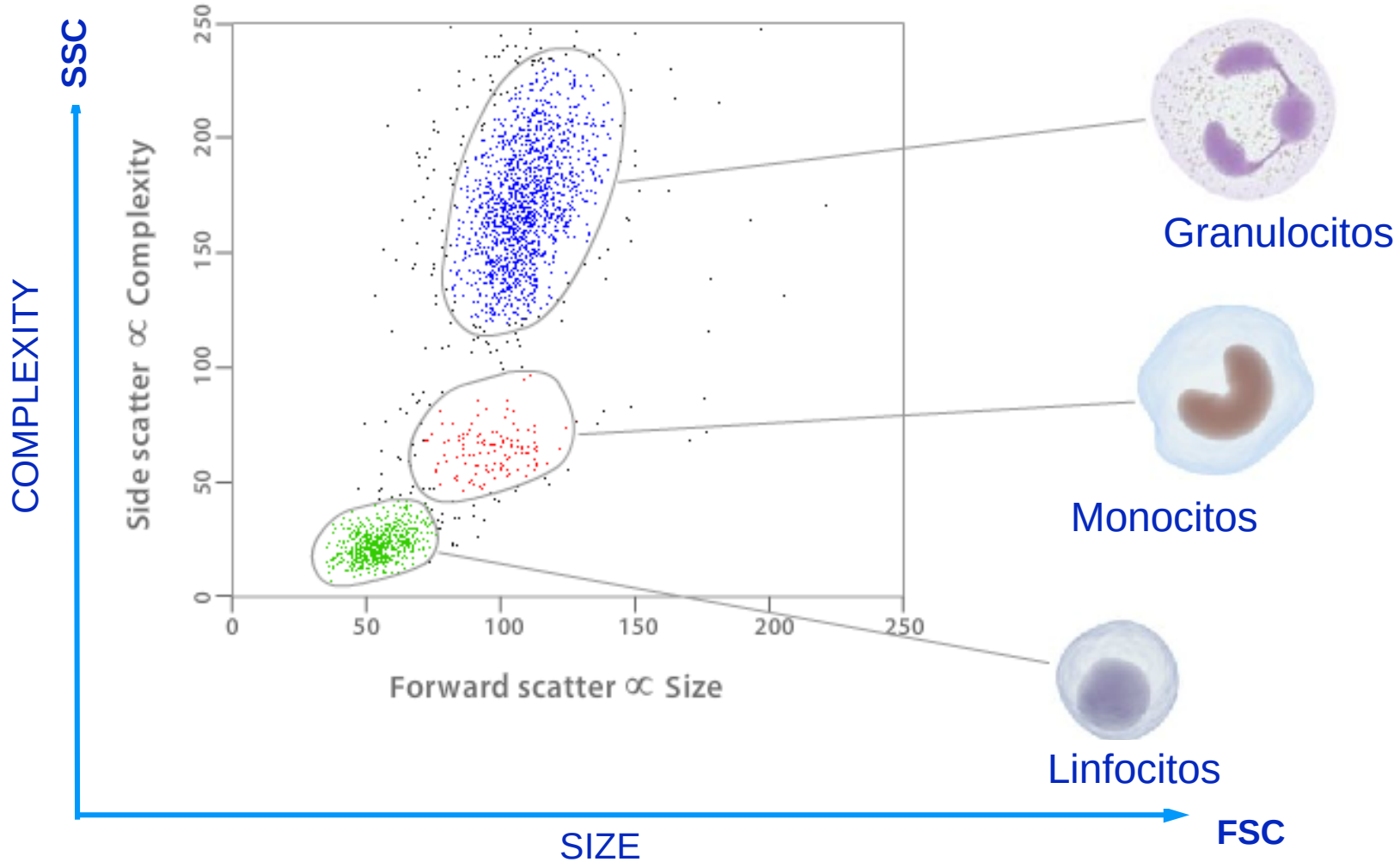
- Lymphocyte phenotype
  - Parameters
  - Staining
  - Markers populations
  - Advanced phenotype
- Lymphocyte function
  - Lymphocyte proliferation
  - Oxidation
  - Phosphorylation
  - Degranulation Basophils/NK
  - Cytotoxicity

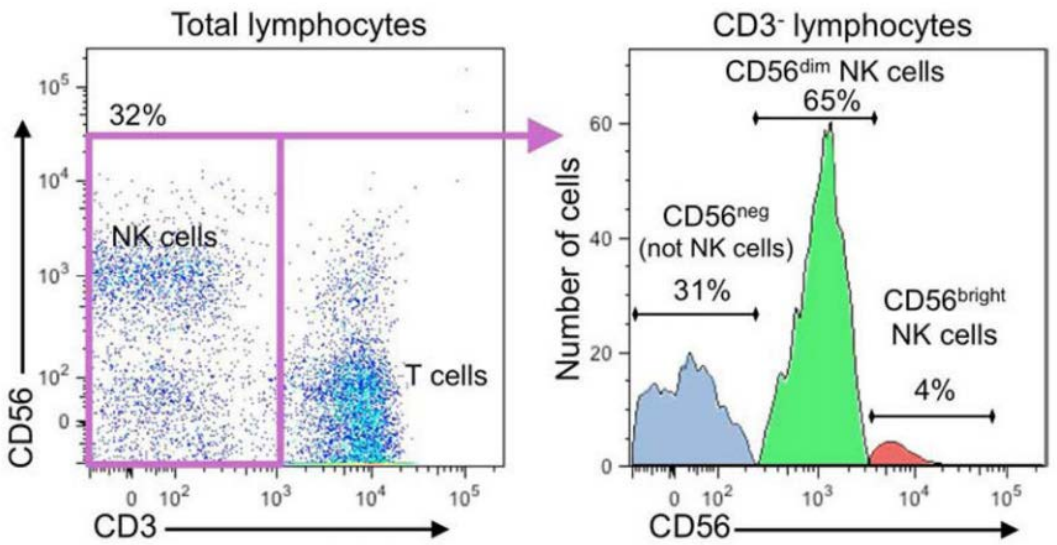
Flow Cytometry

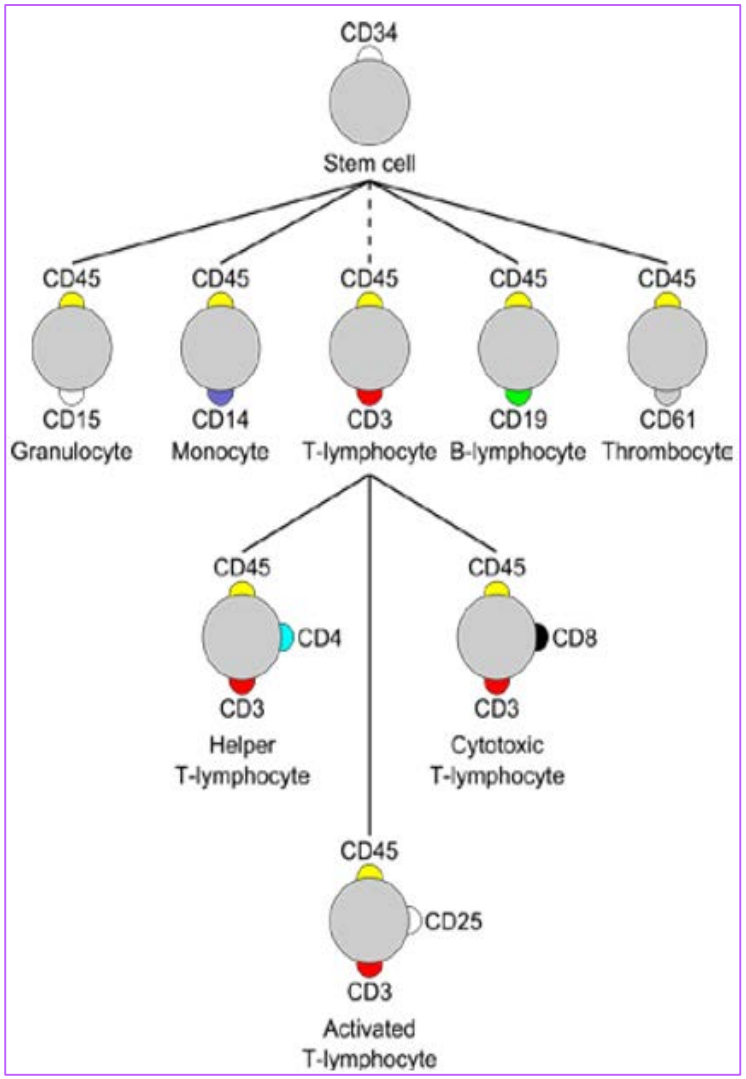


Cellular immunology

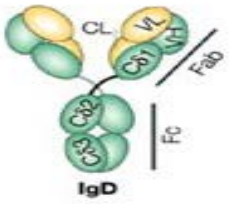


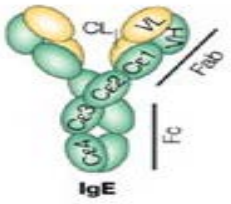
Fluorescent Antibodies

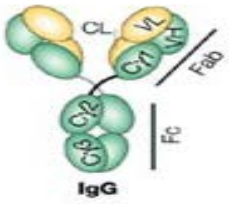




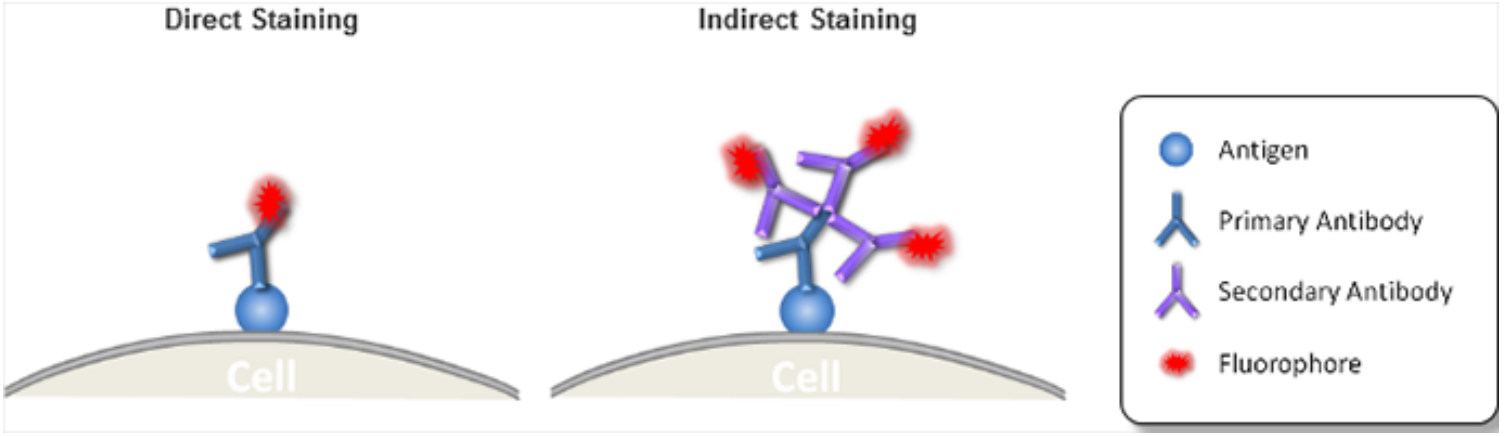
Fluorochrome
Clone



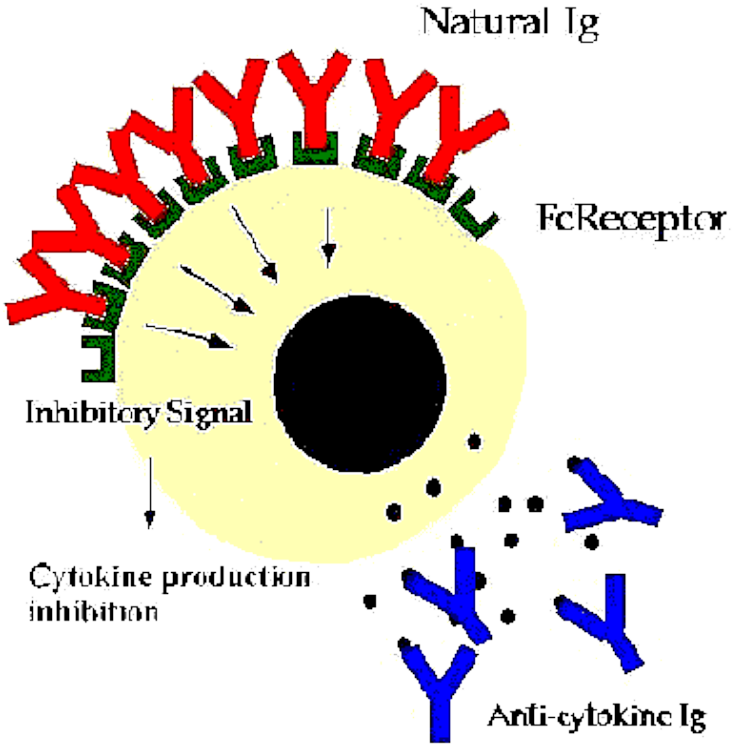


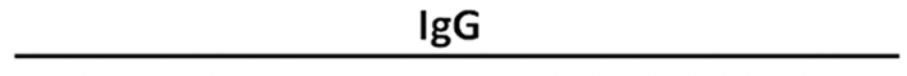


Staining methods



Fc Blocking Controls

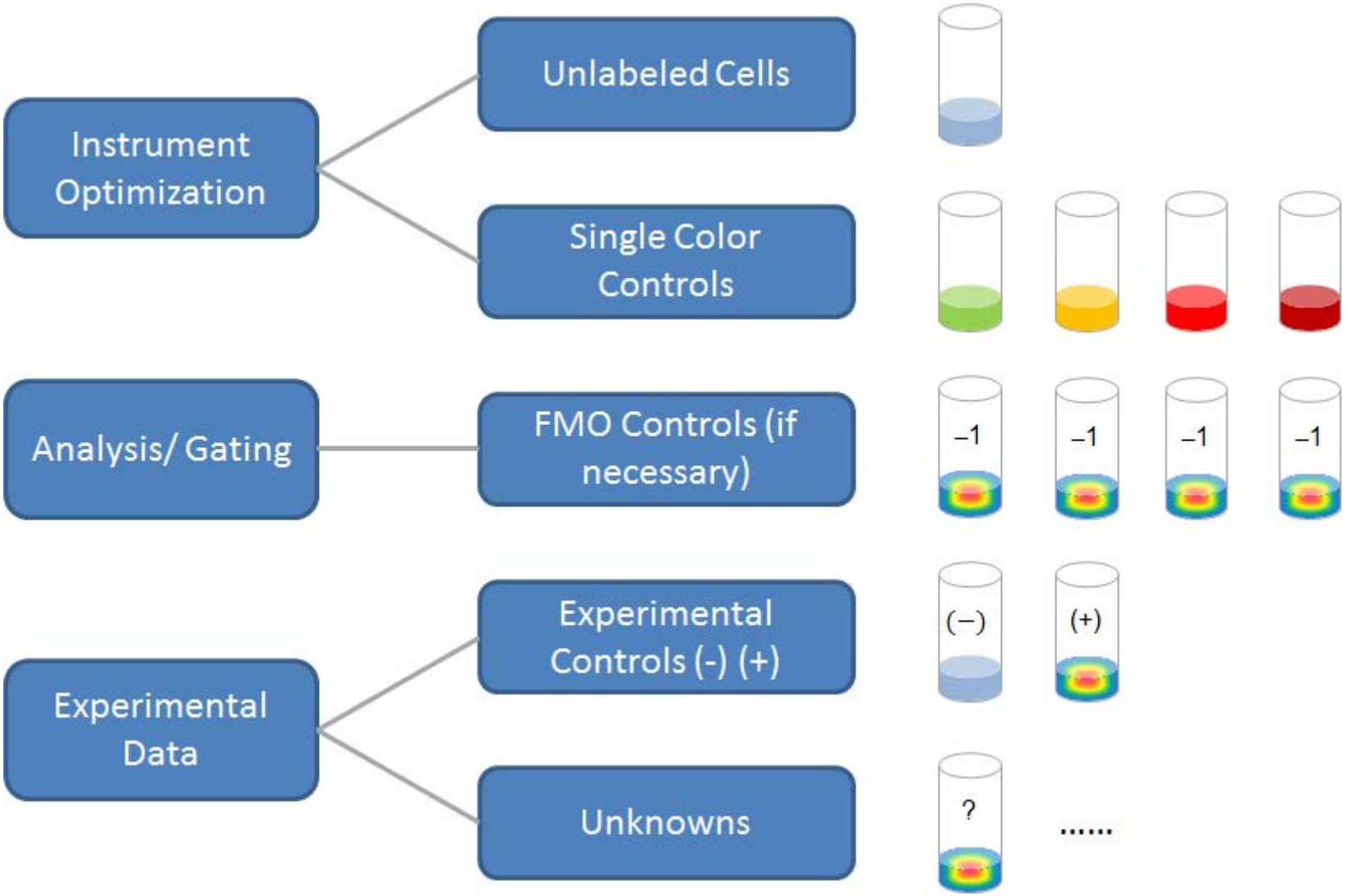




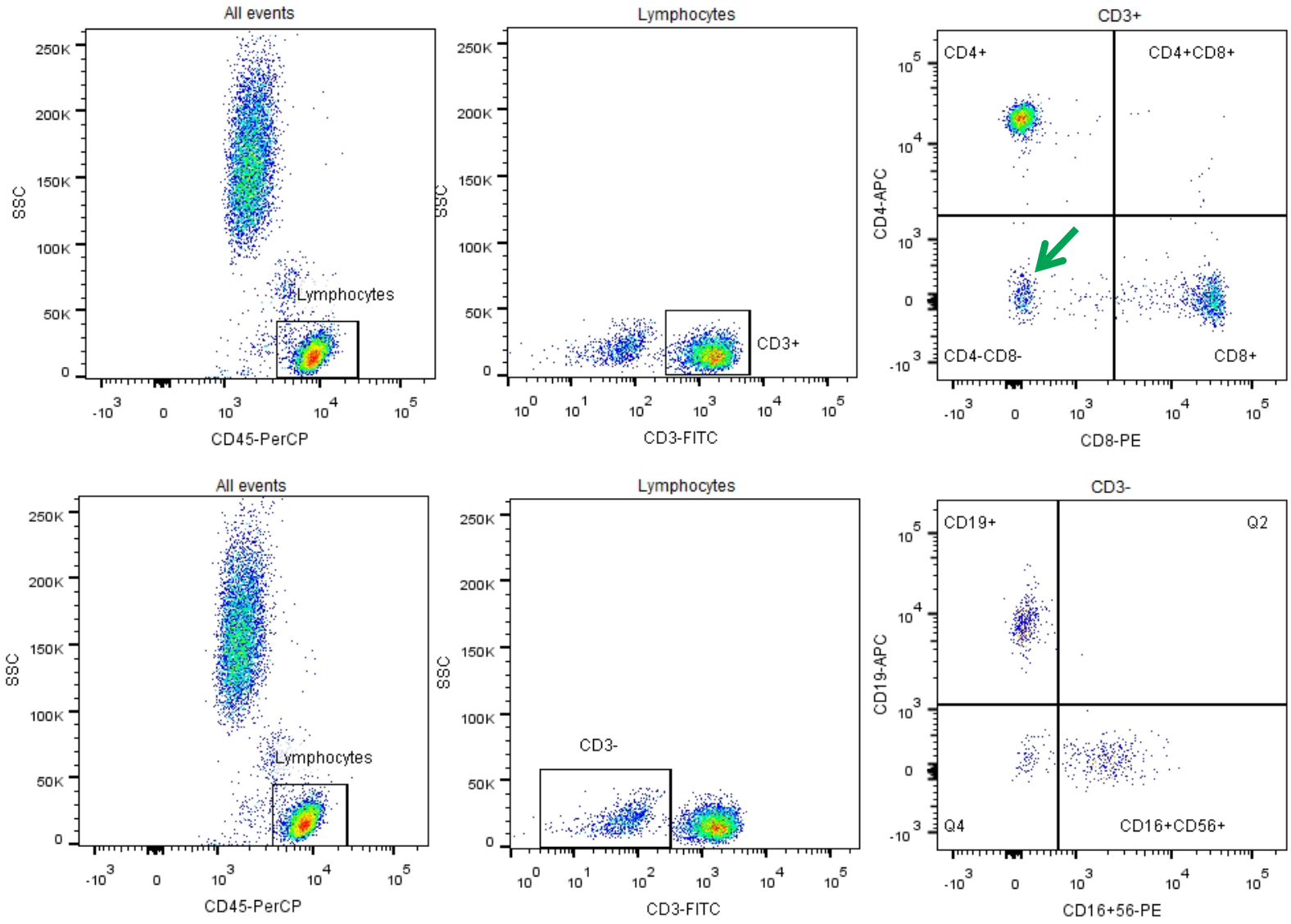
	High affinity		Low affinity		High & Low		Low affinity	High affinity pH<6.5
Function	Activation	Activation	Inhibition	Activation	Activation		?	Recycling Transport Ag uptake…
Variant		*H_{131}*	*R_{131}*			*V_{158}*	*F_{158}*	
IgG subclass binding	**1**	1	1	1	1	1	1	**1**
	-	2	(2)	(2)	(2)	(2)	(2)	-	**2**
	3	3	3	3	3	3	3	3	3
	4	4	4	4	4	4	4	4	**4**

Nonspecific binding of antibodies to leukocytes is mainly due to binding to Fc receptors (FcRs, CD16, CD32, CD64).

Highest expression on monocytes and macrophages


Instrument Optimization
Unlabeled Cells
Single Color Controls
Analysis/ Gating
FMO Controls (if necessary)
Experimental Data
Experimental Controls (-) (+)
Unknowns
–1
–1
–1
–1
(–)
(+)
?
.....

Flow cytometry immunophenotyping



T+ B+ NK ≅ 100%

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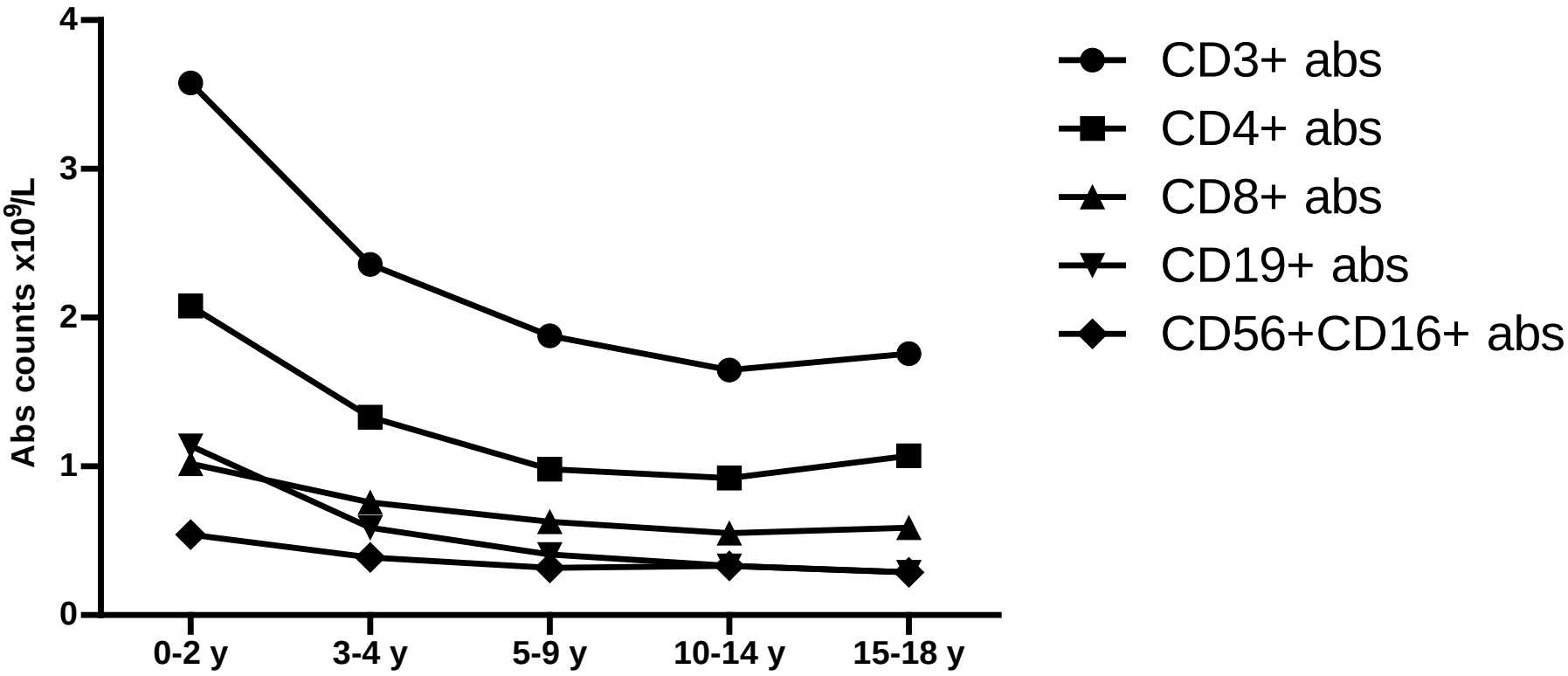
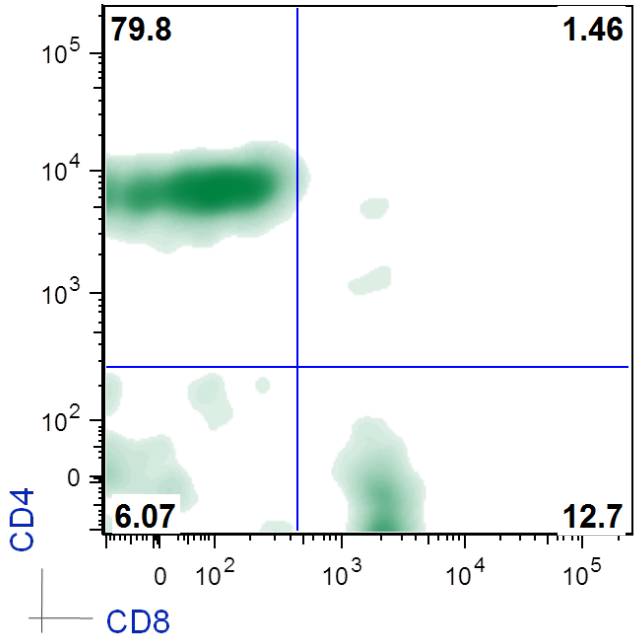
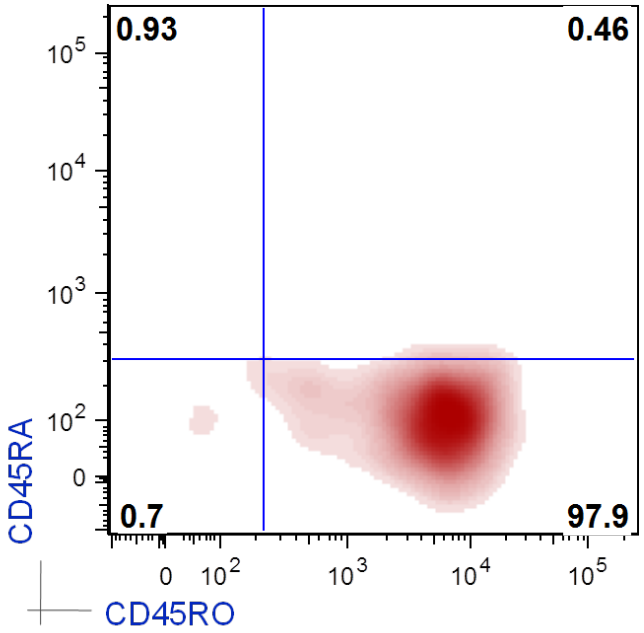
Age-specific reference ranges



Garcia-Prat M. et al. 2018

Naïve/Memory cells (CD45RA+/CD45RO+)





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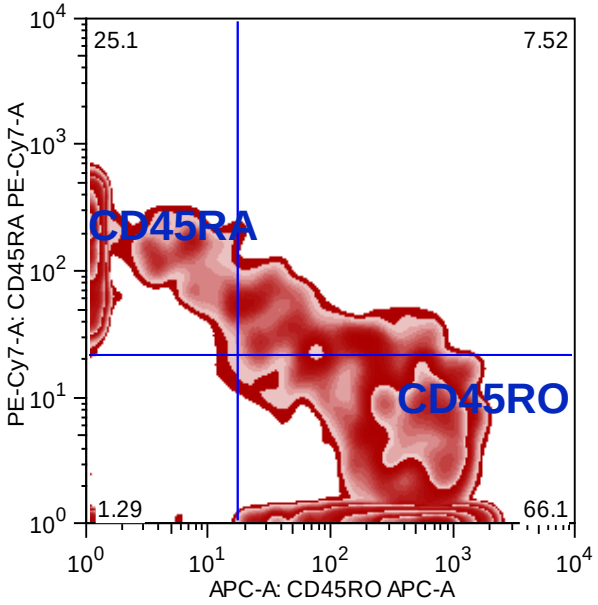
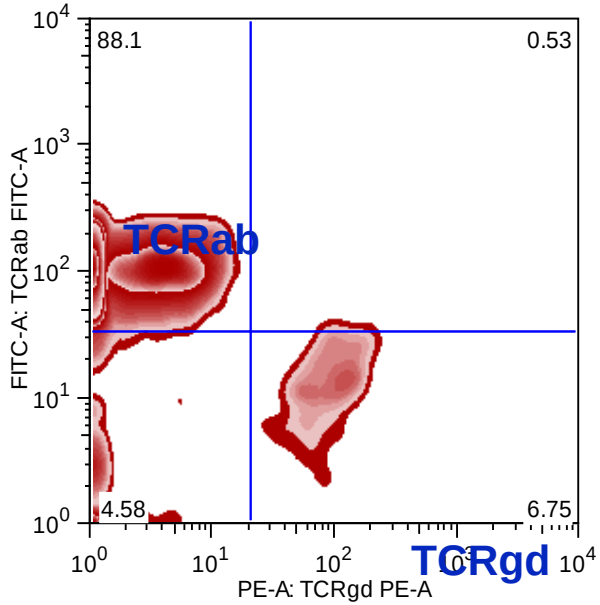
Naïve/Memory cells (CD45RA+/CD45RO+)





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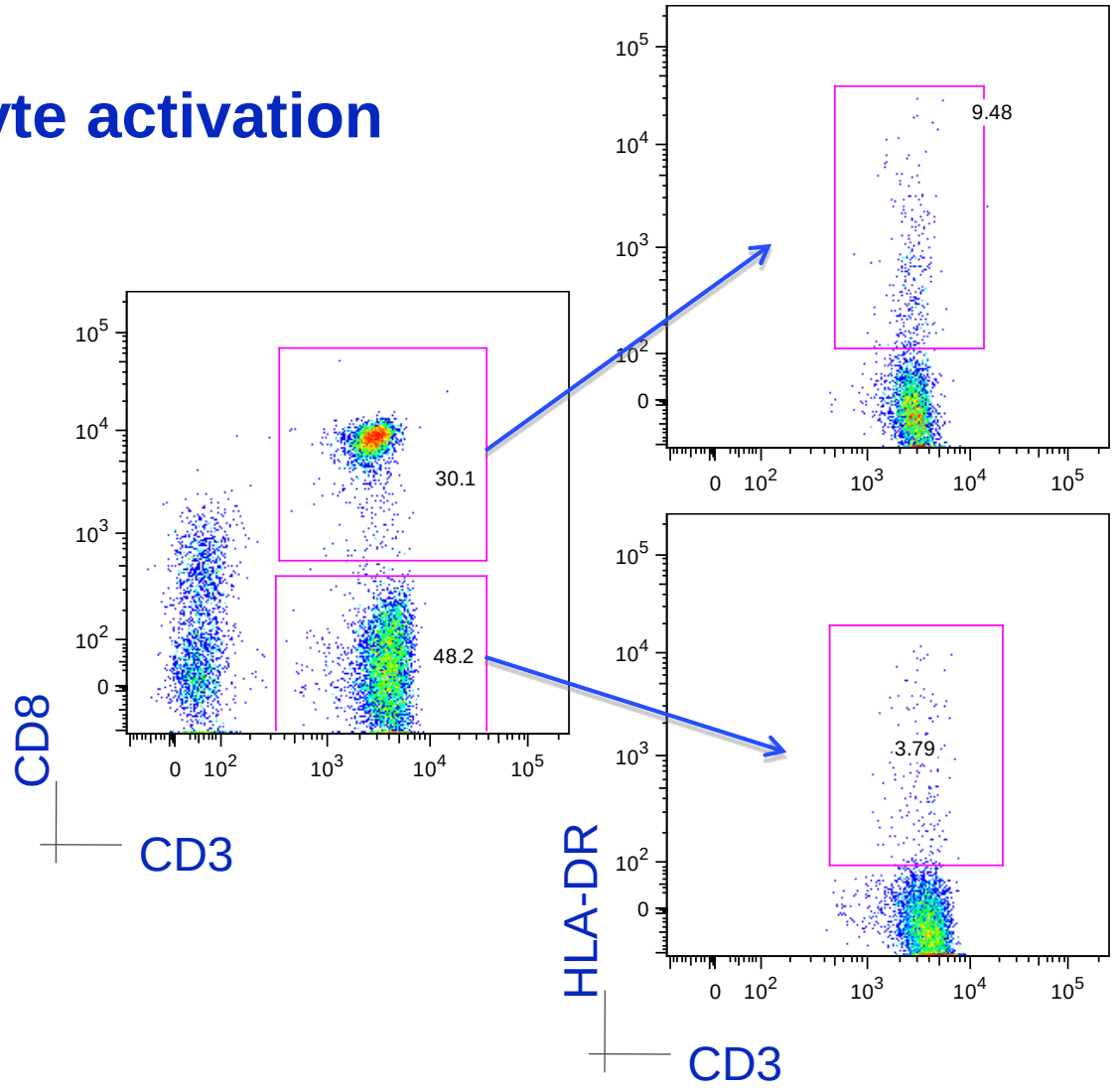
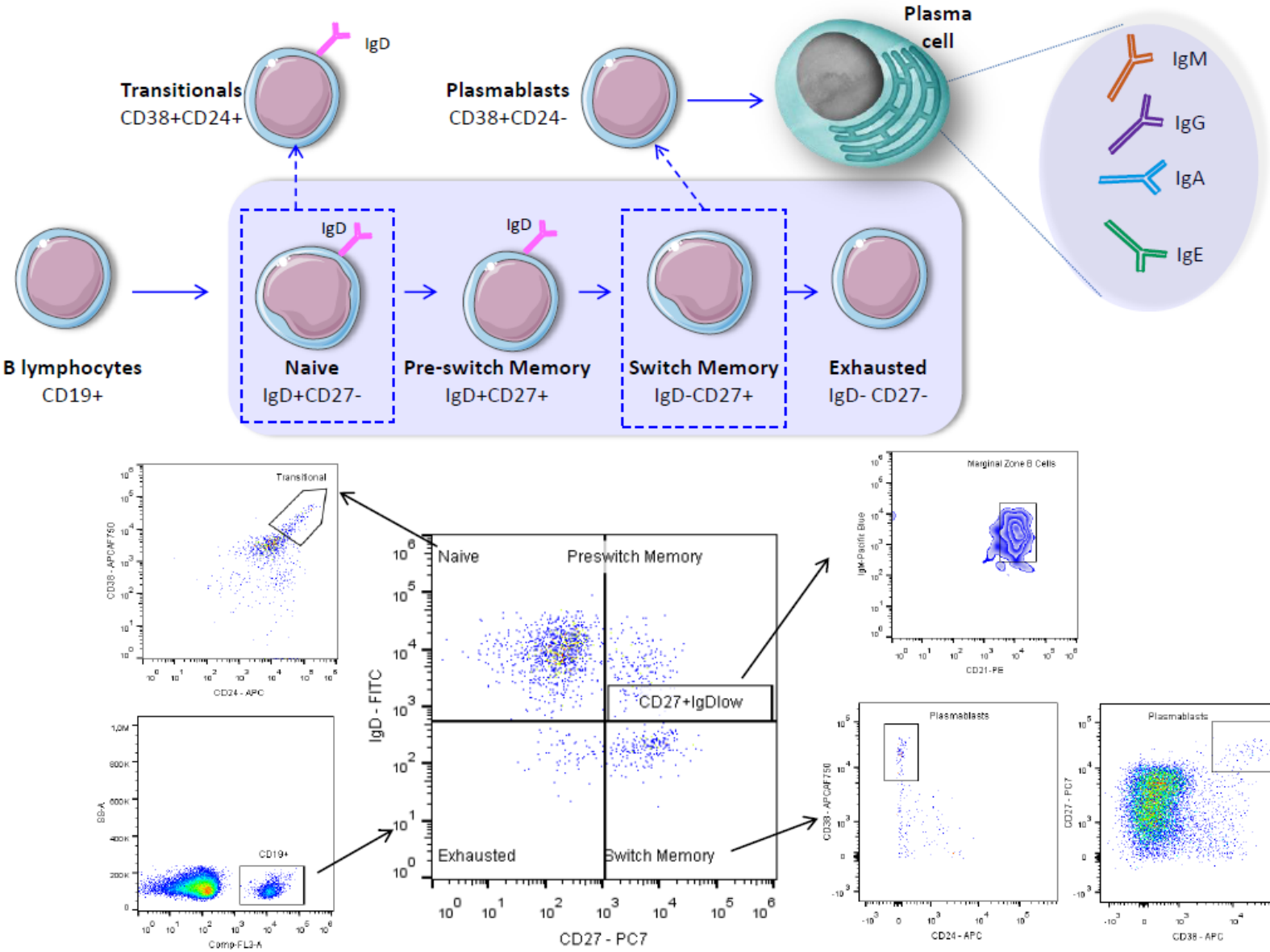
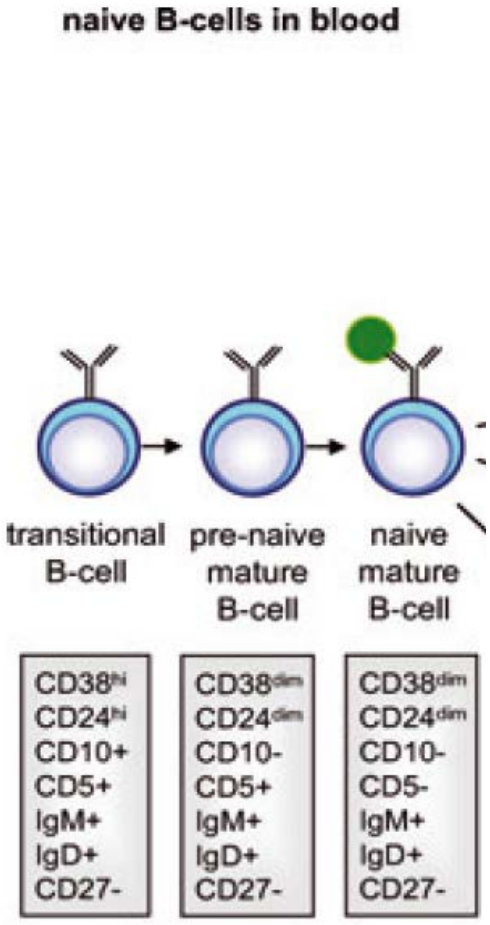
Lymphocyte activation

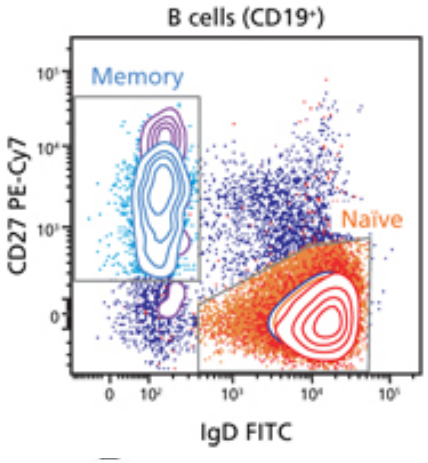


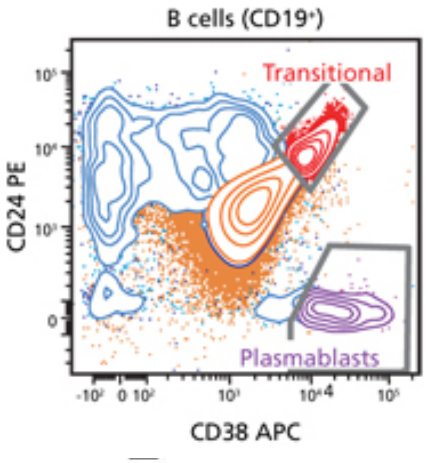
Advanced immunophenotyping

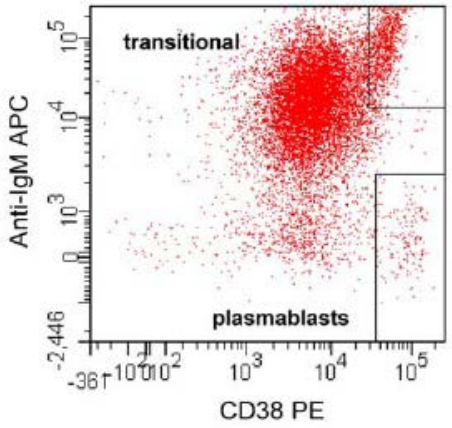


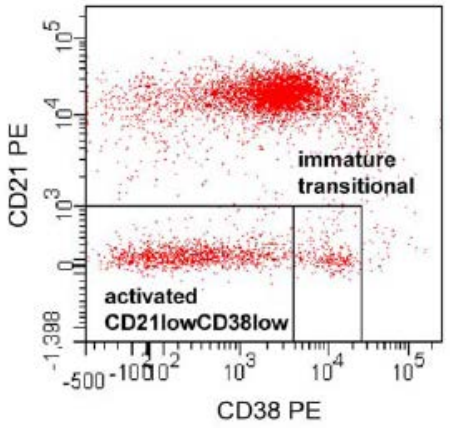
B lymphocytes immunophenotype

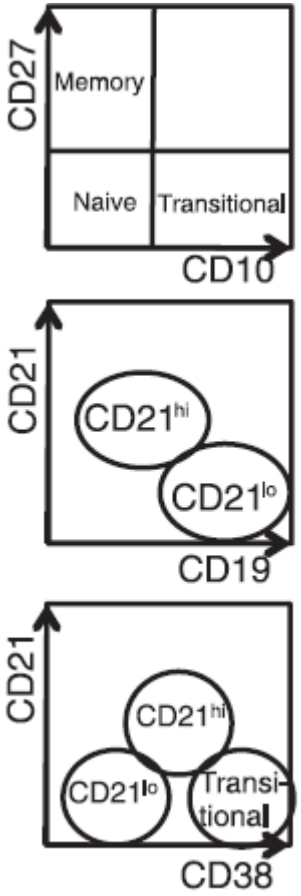

naive B-cells in blood
transitional B-cell
pre-naive mature B-cell
naive mature B-cell
CD38hi CD24hi CD10+ CD5+ IgM+ IgD+ CD27-
CD38dim CD24dim CD10- CD5+ IgM+ IgD+ CD27-
CD38dim CD24dim CD10- CD5- IgM+ IgD+ CD27-



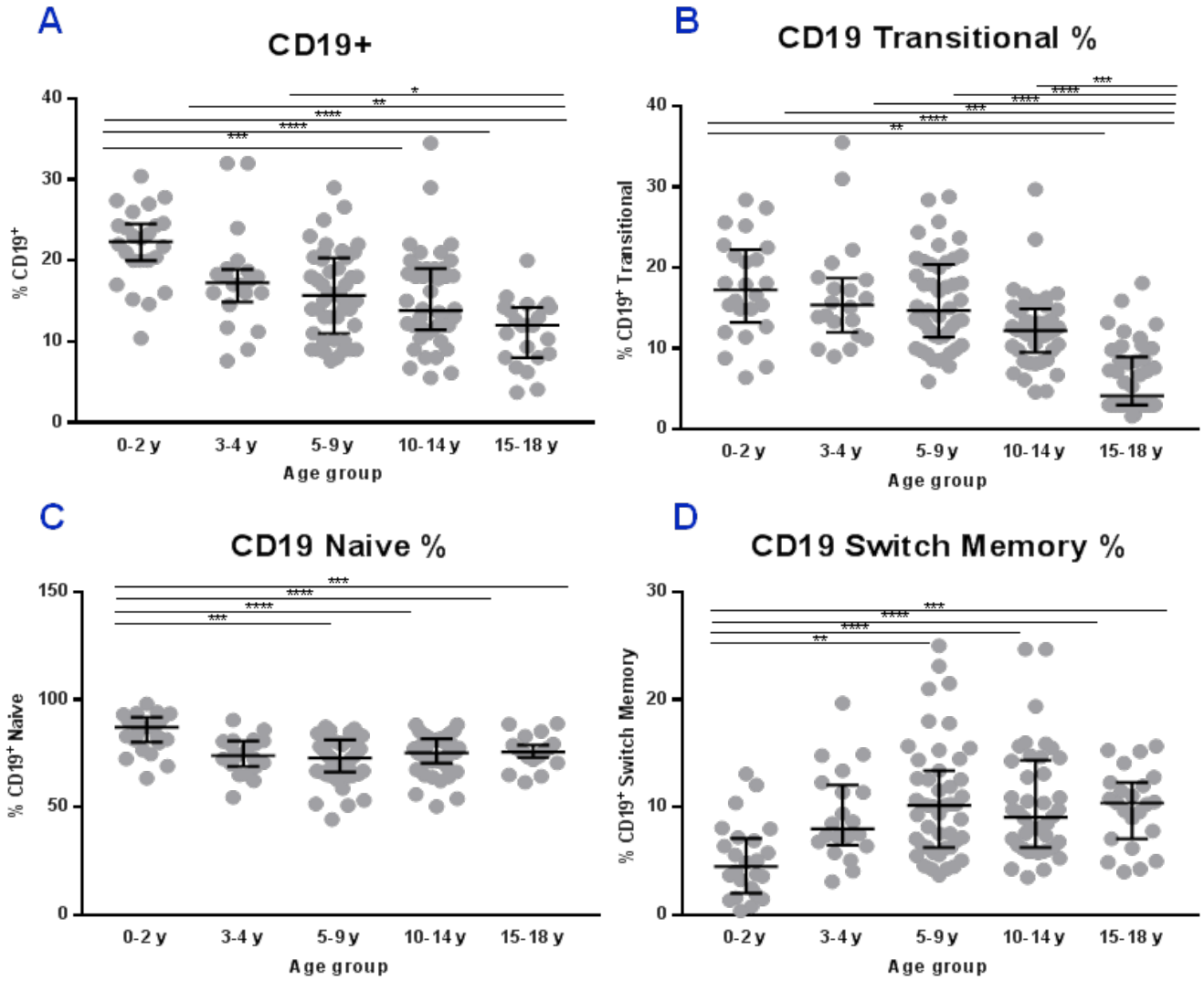








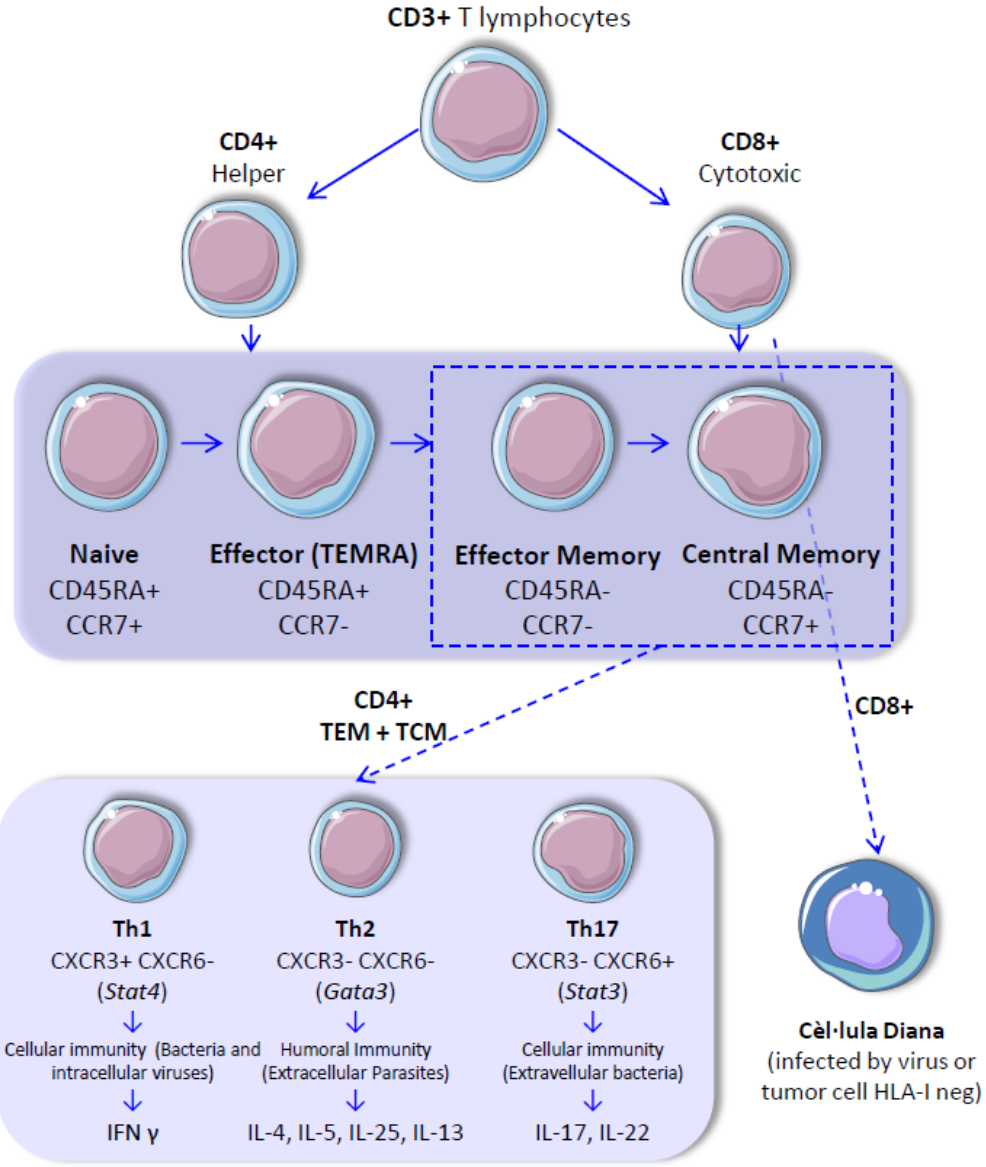
Advanced immunophenotyping

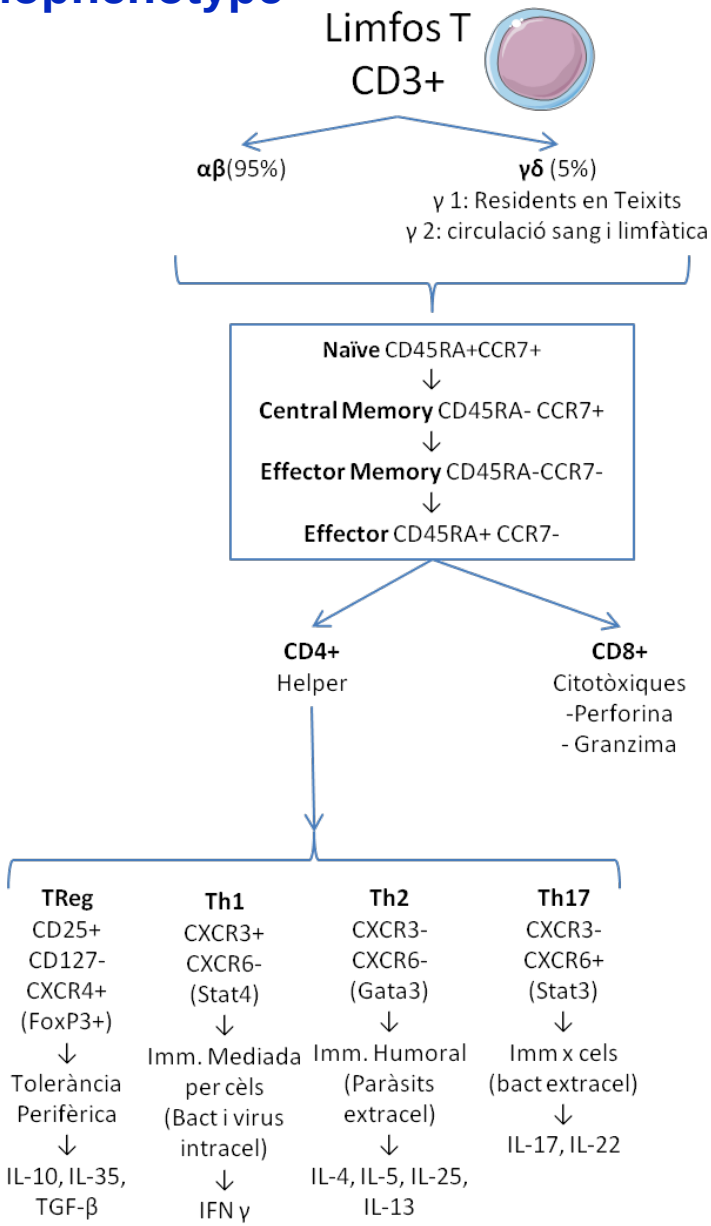


Garcia-Prat M et al. 2018

Vall d'Hebron

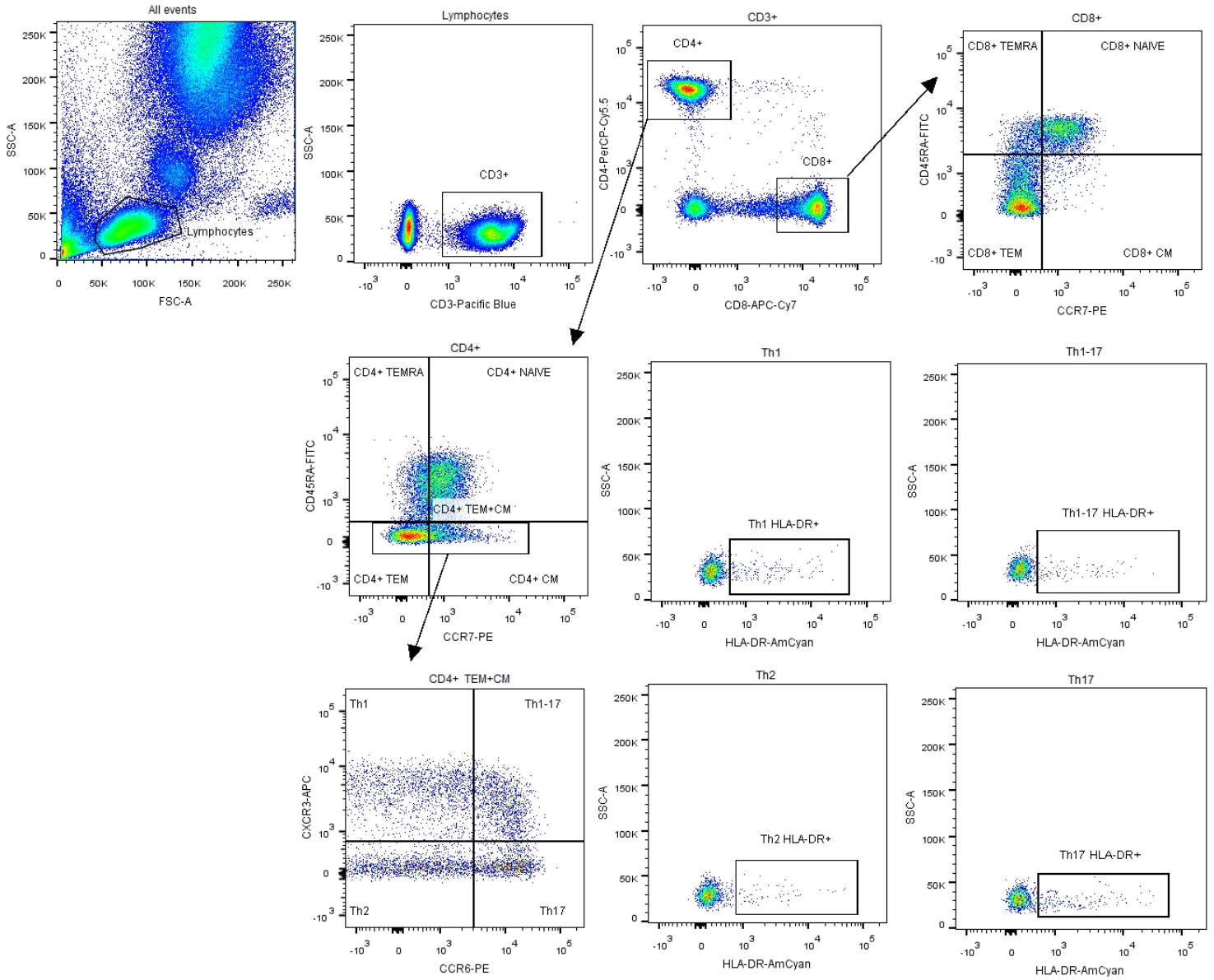
T lymphocytes immunophenotype





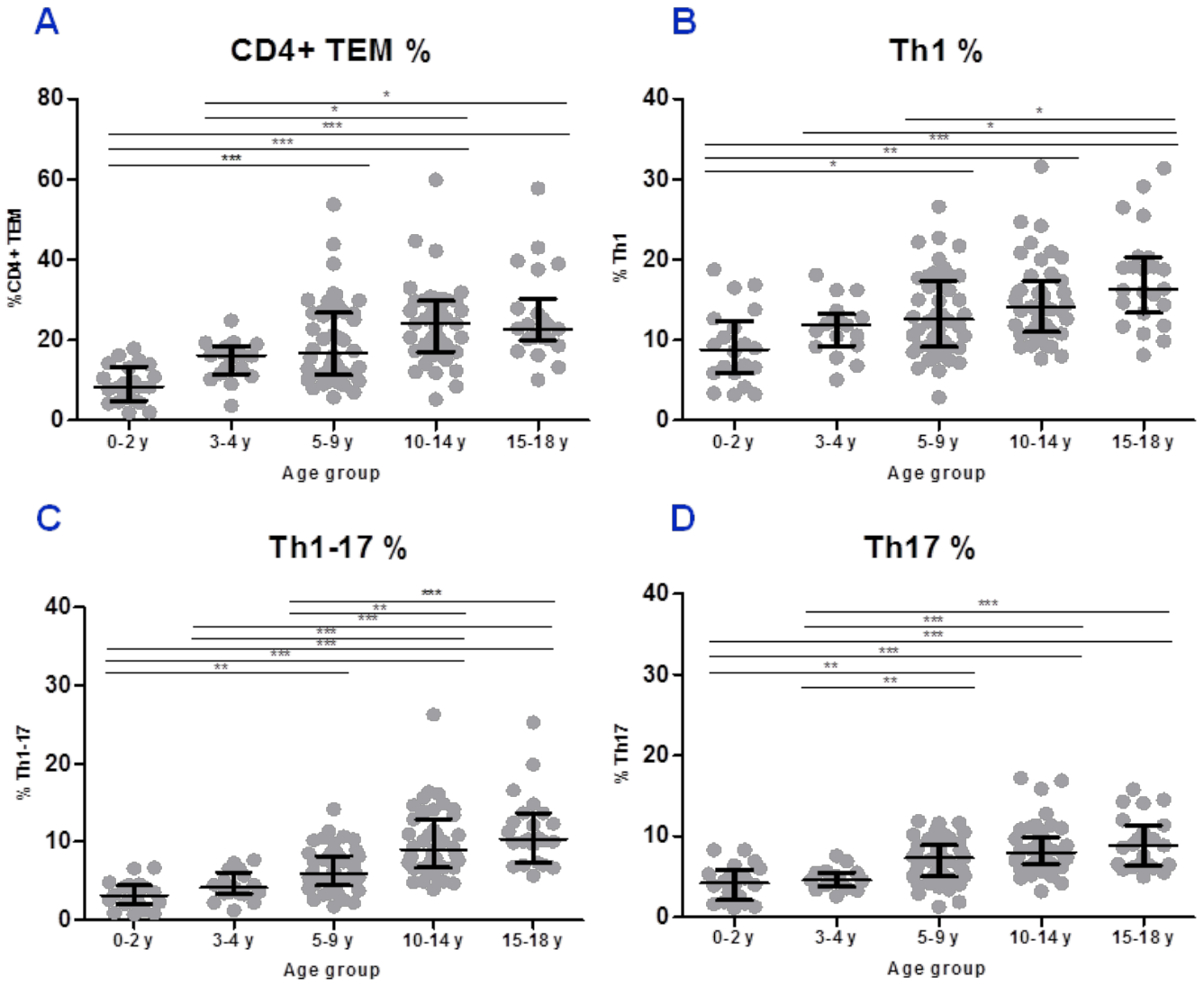
Vall d'Hebron

Advanced immunophenotyping



Vall d'Hebron

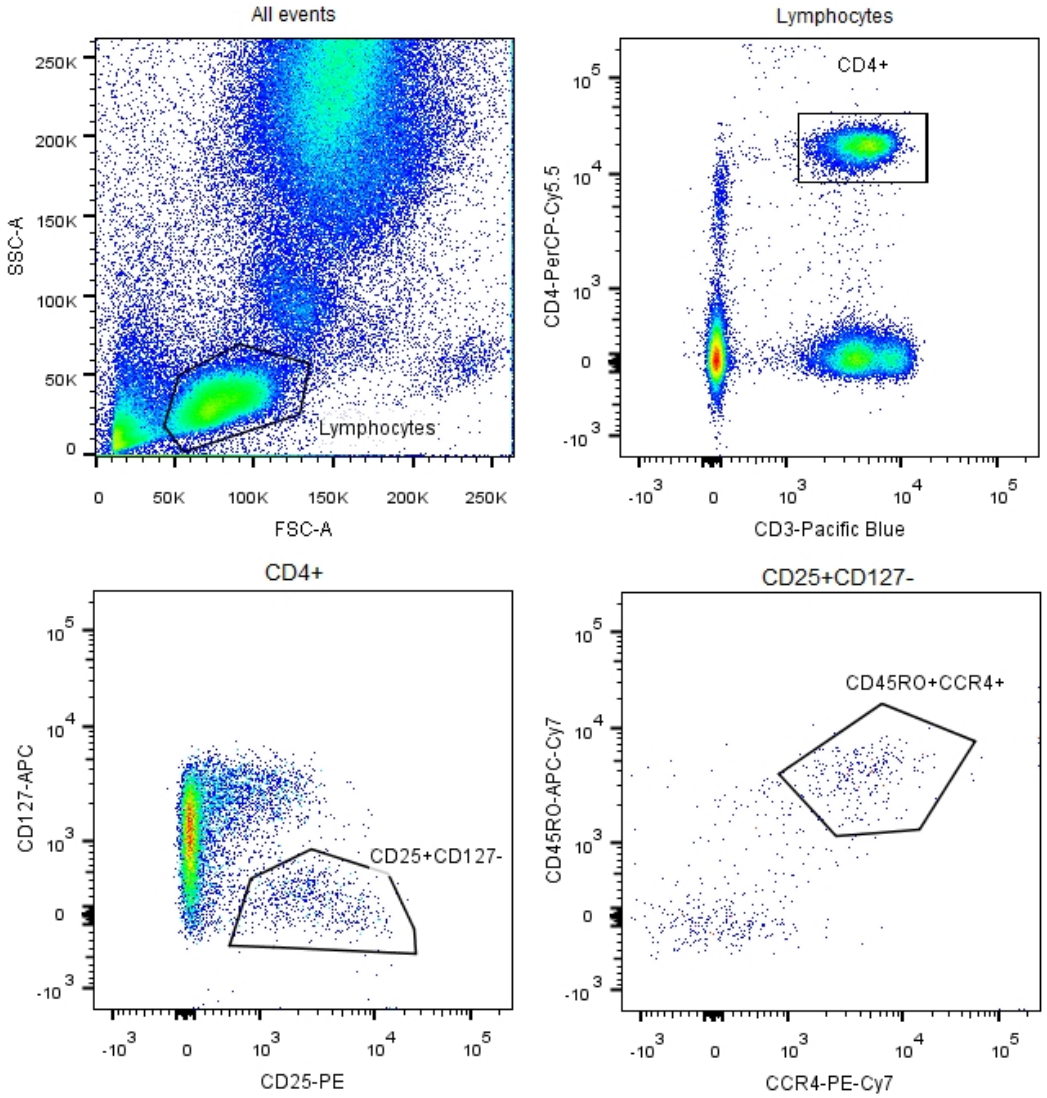
T lymphocytes immunophenotype

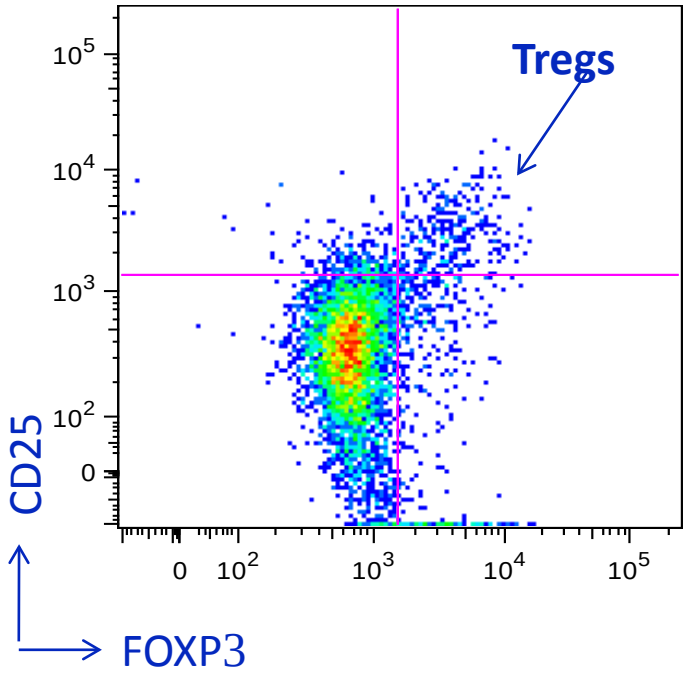


Garcia-Prat M. et al. 2018

Vall d'Hebron

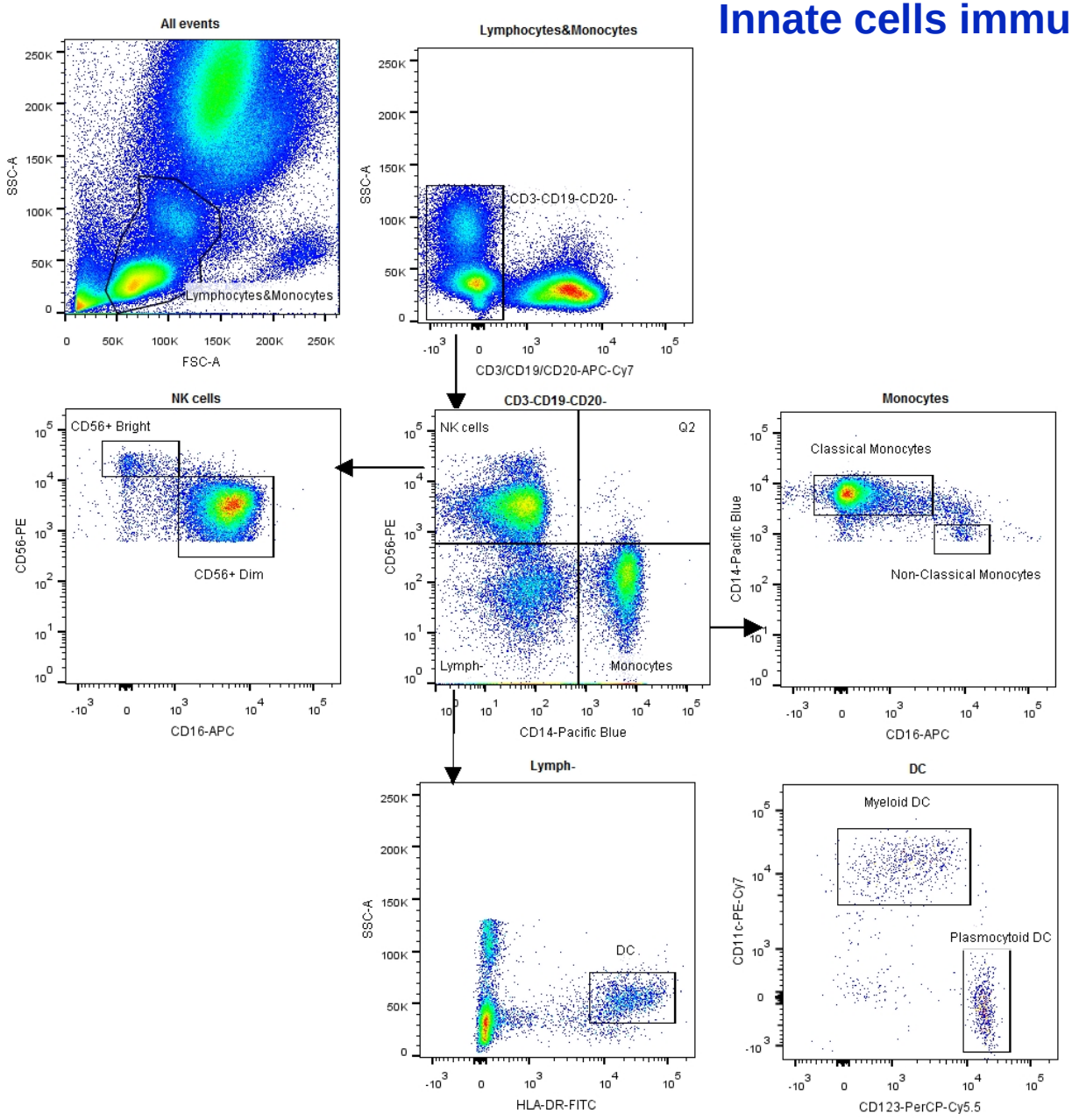
Advanced immunophenotyping: Tregs





Vall d'Hebron

Innate cells immunophenotype



CD3-CD19-

Monòcits
CD3-CD14+CD56-

Classical	**Non-Classical**
CD14+	CD14+
CD16-	CD16+

NK
CD3- CD16+ CD56+

Bright (Reguladores)
↑CD56
↓CD16

+ edat **-edat**

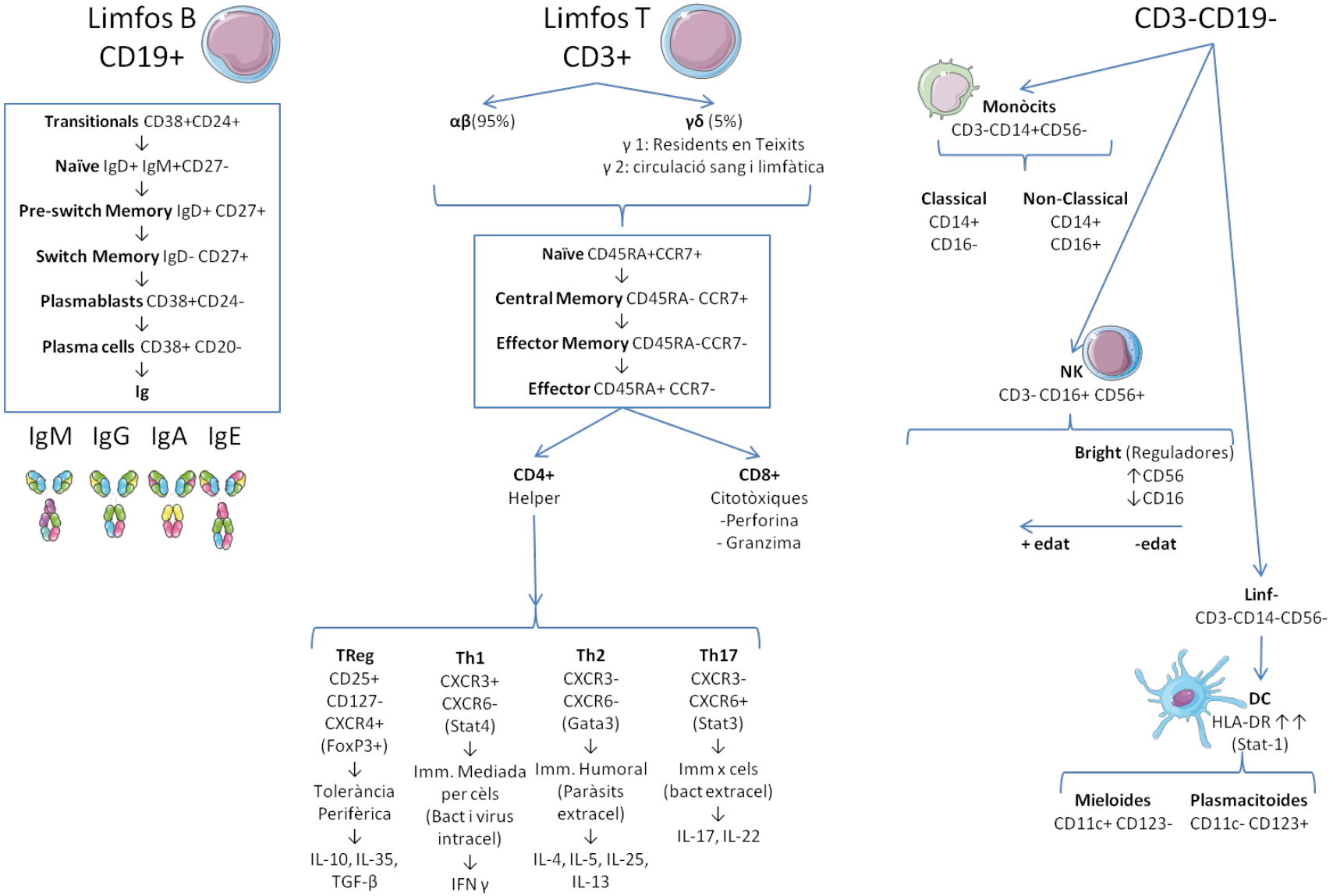
Linf-
CD3-CD14-CD56-

DC
HLA-DR ↑↑
(Stat-1)

Mieloides	**Plasmacitoides**
CD11c+ CD123-	CD11c- CD123+

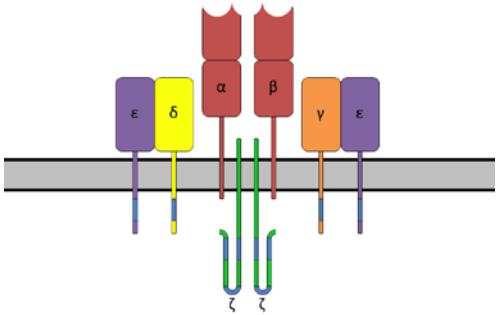
Vall d'Hebron

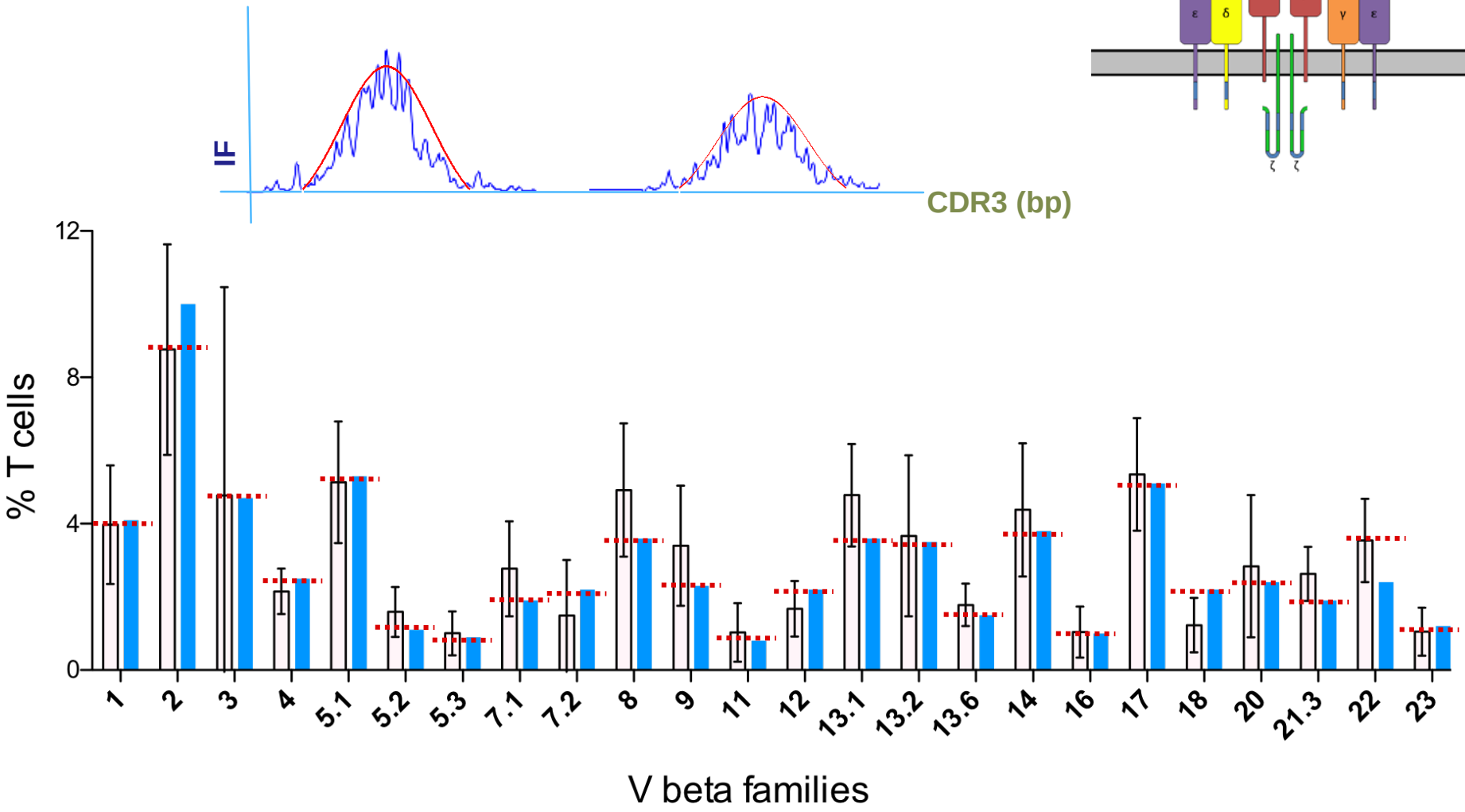
Advanced immunophenotyping



Vall d'Hebron

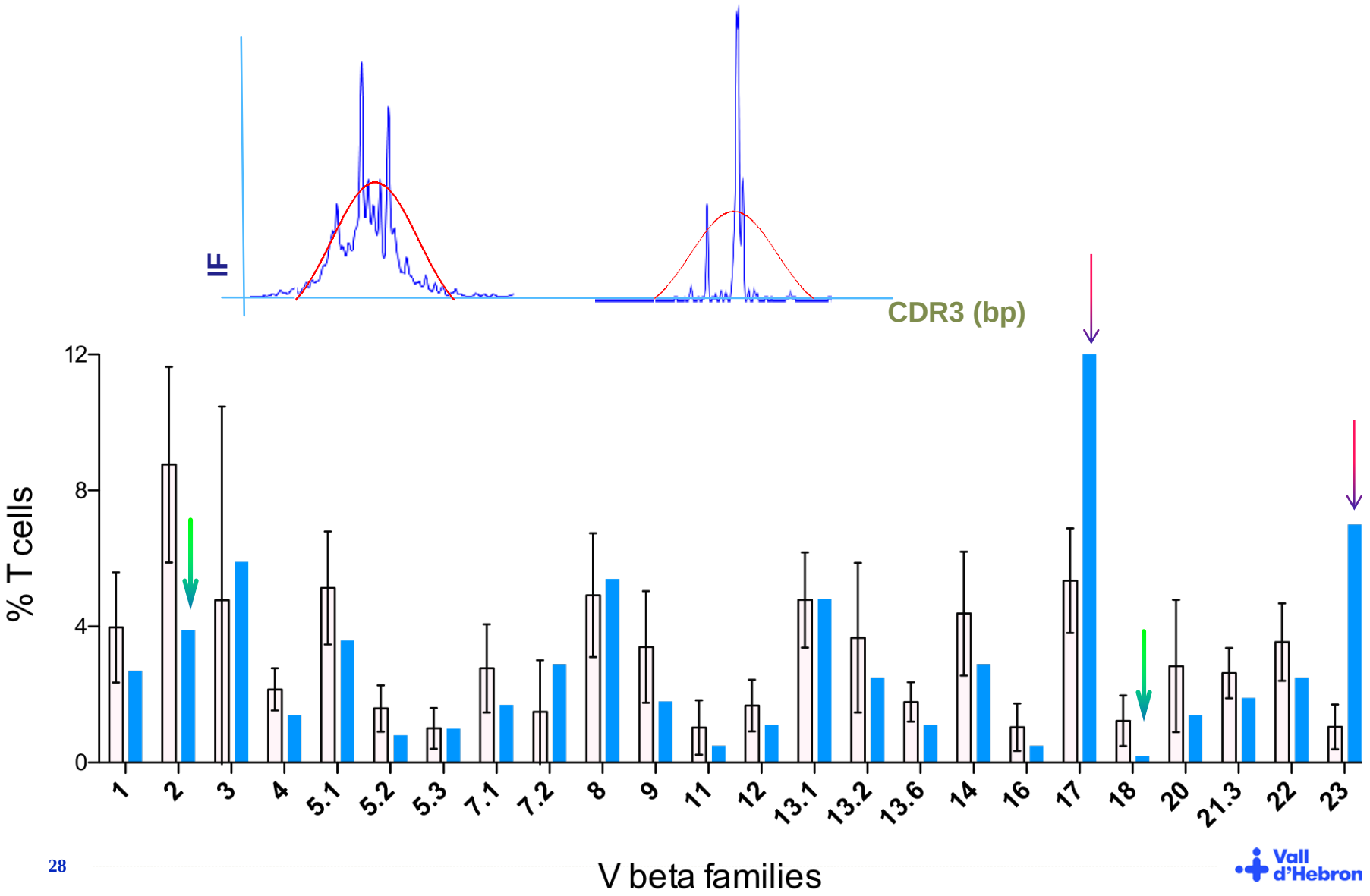
T Cell repertoire





Vall d'Hebron

T Cell repertoire



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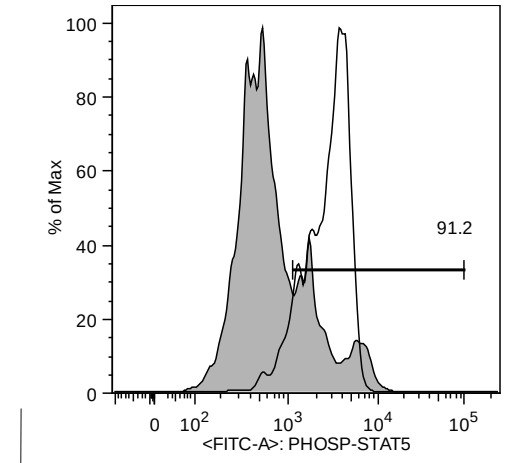
Functional Analysis

- CD40 binding to CD40 ligand (done routinely with **CD40L protein expression**)
- **IκBα phosphorylation/degradation** assay (screening for EDA-ID)
- **Lymphocyte apoptosis**, Fas-mediated (screening test for ALPS)
- Neutrophil **oxidative burst assay** (screening test for chronic granulomatous disease)
- **NK cell/cytotoxic T-cell degranulation** assay (CD107)
- **STAT1 phosphorylation** in response to interferon gamma (screening test for interferon-gamma receptor deficiency)
- STAT3 phosphorylation in response to IL-6 (HIES screening)
- STAT5 phosphorylation in response IL2 (SCID screening)



STAT5-P

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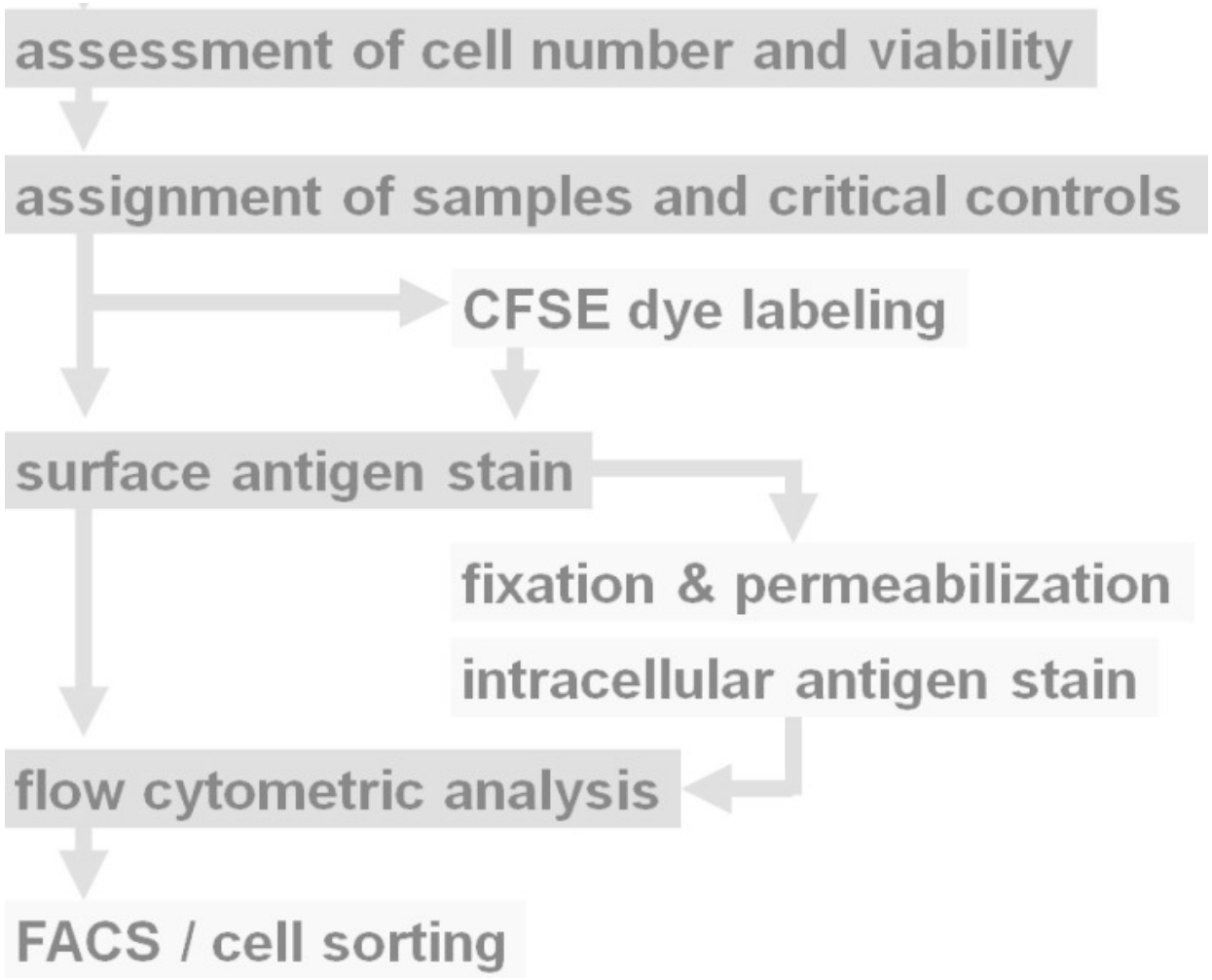
Planning your experiment



Lymphocyte proliferation assays

PBMCs
Peripheral
Blood
Mononuclear
Cells

(Limfòcits + Monòcits)

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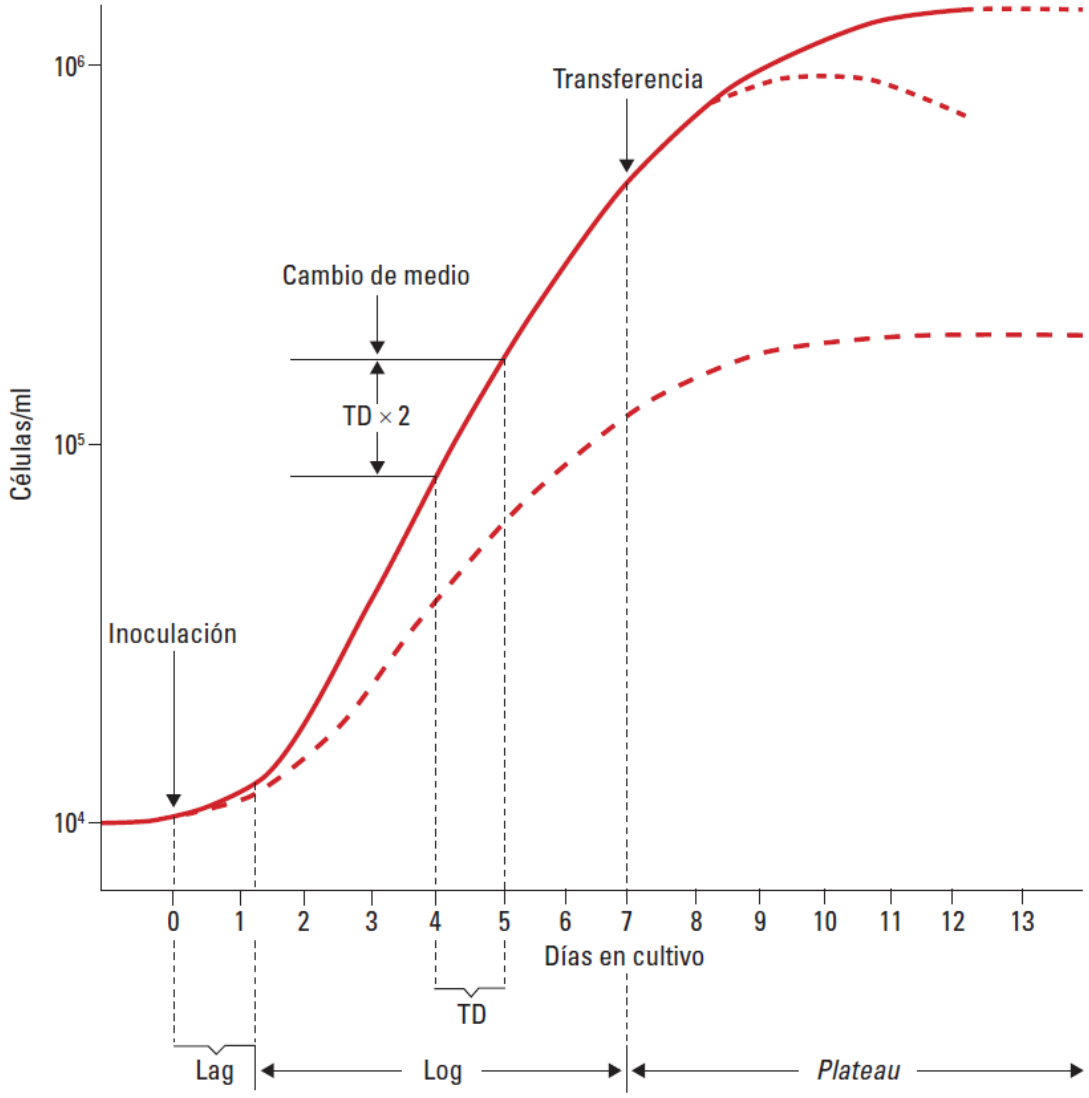
Curva de Crecimiento (líneas estables): Fases del cultivo



- **Fase de latencia** (período lag). Fijación al sustrato e inicio del ciclo celular.
- **Fase de crecimiento exponencial**. El número de células se duplica aproximadamente cada 24 horas.
- **Fase de confluencia**. Las células del cultivo, que se ha saturado, dejan de dividirse (monocapa: inhibición por contacto; suspensión: consumo del medio).
- **Fase de muerte**. Si el cultivo se prolonga por demasiado tiempo, las células entran en una fase de senescencia que termina con la muerte de las células en cultivo.

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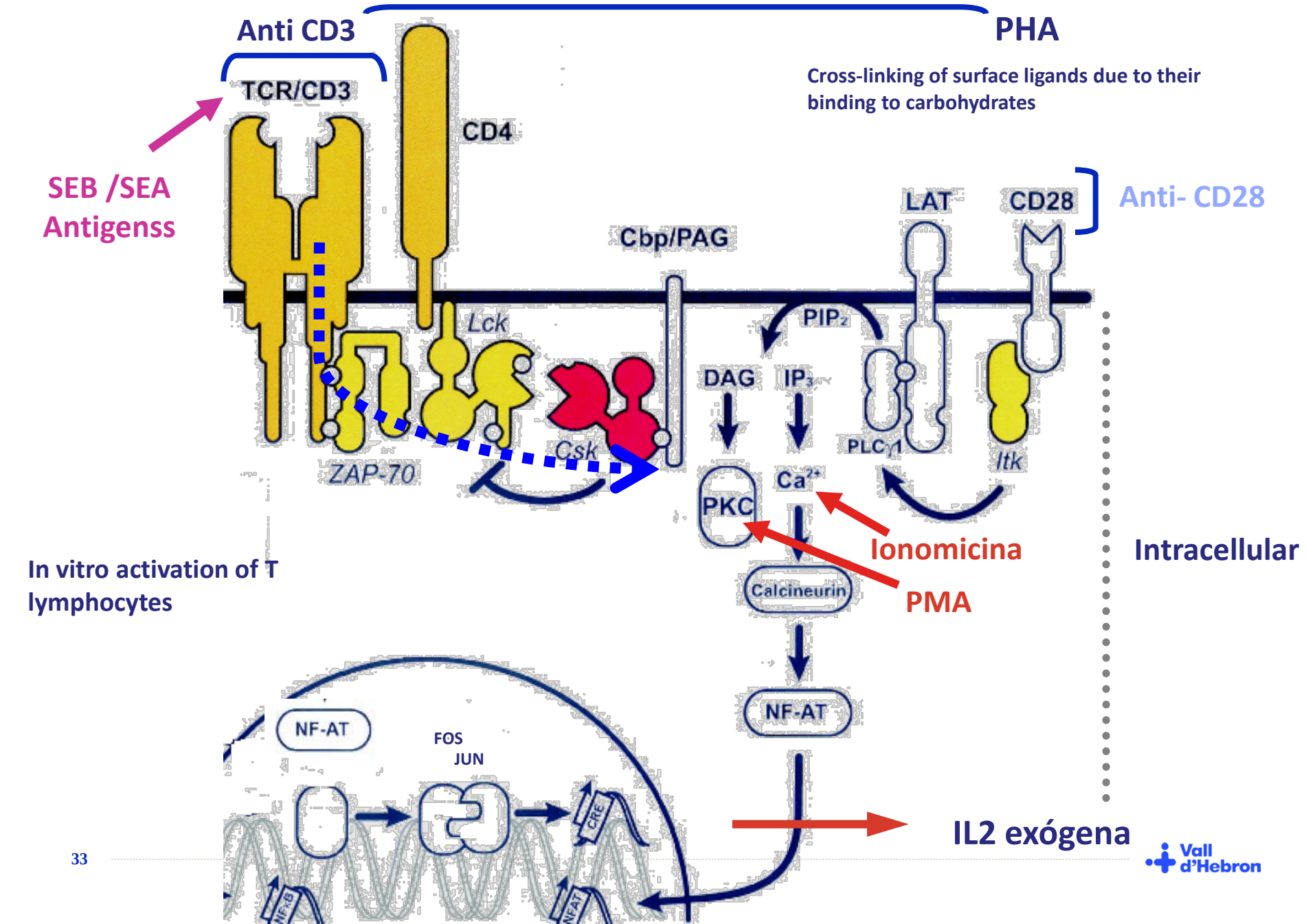
Lymphocyte proliferation assays



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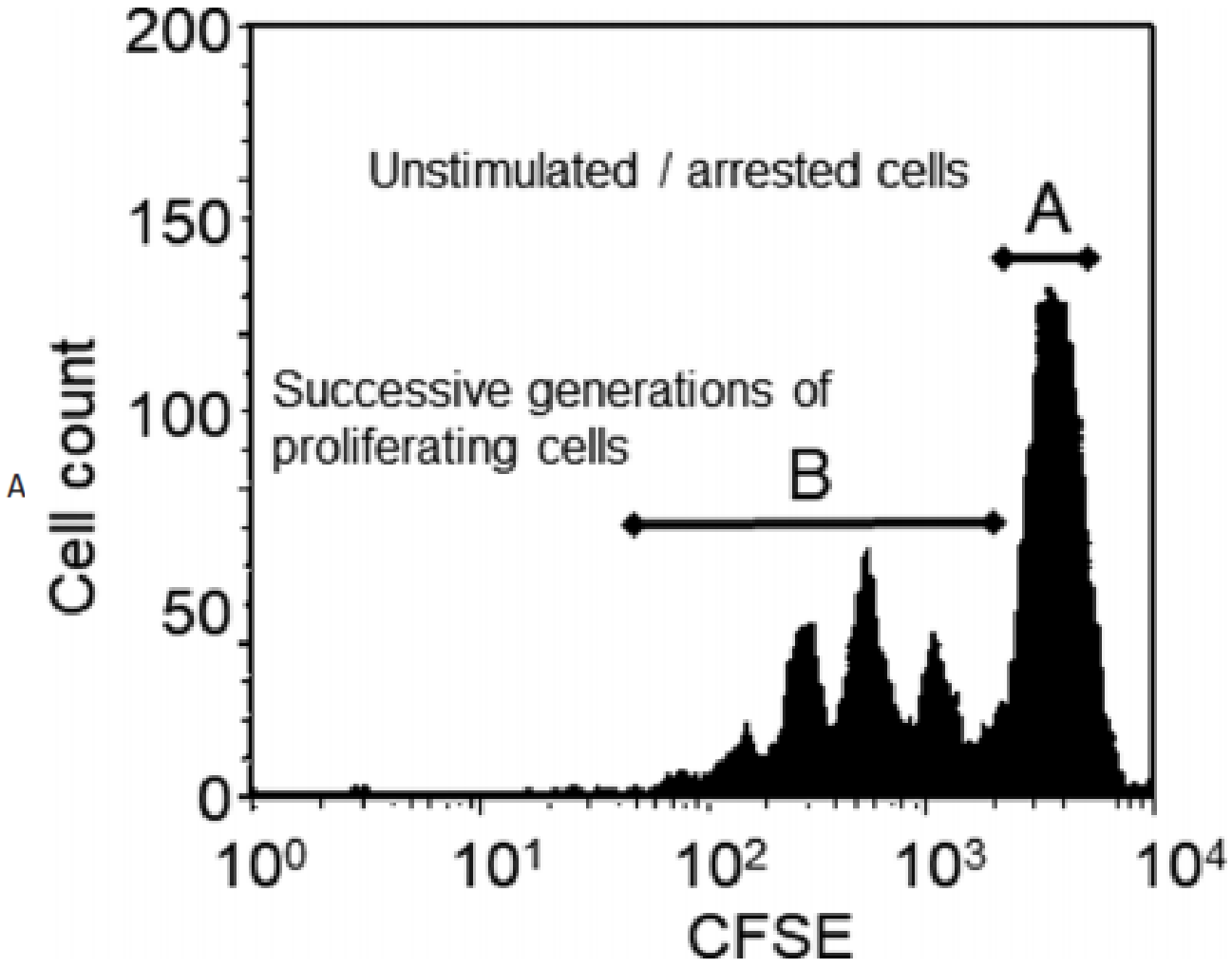
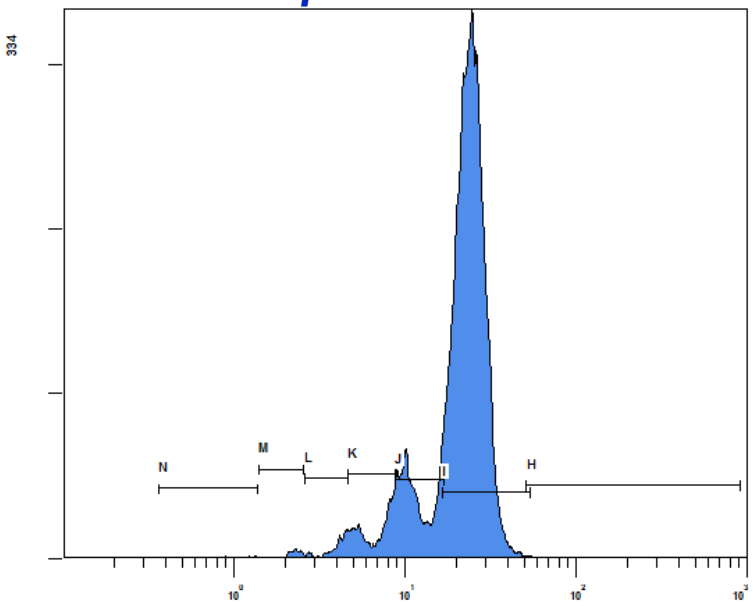
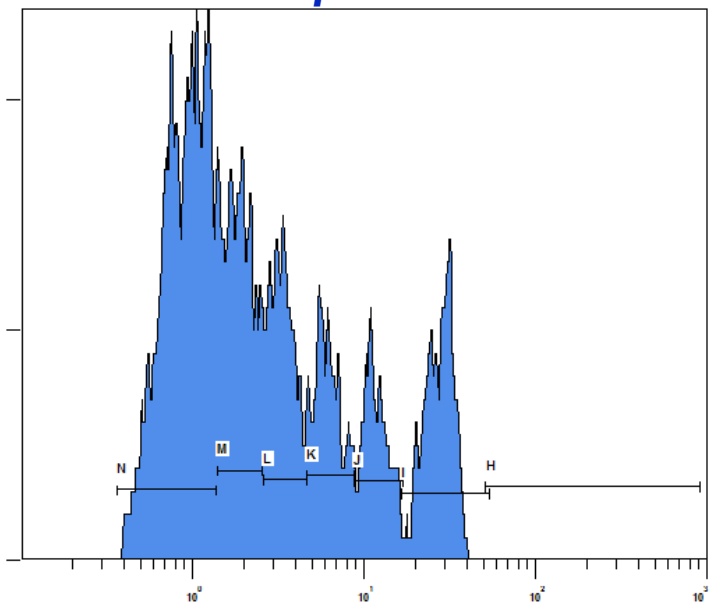
Lymphocyte proliferation assays



Proliferació absent

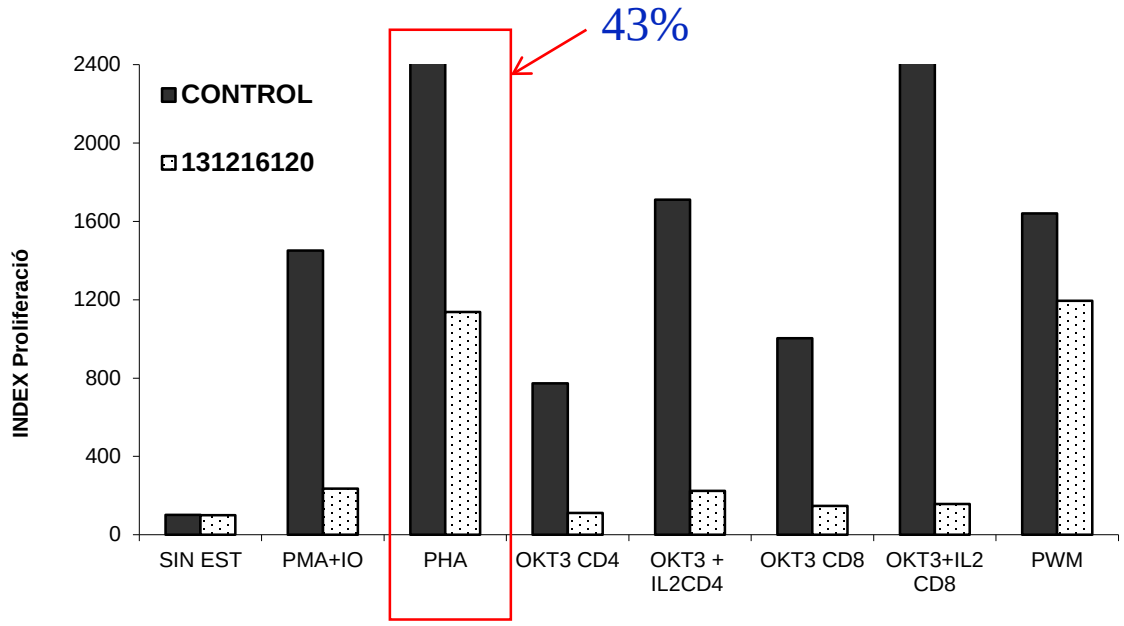


Proliferació OK

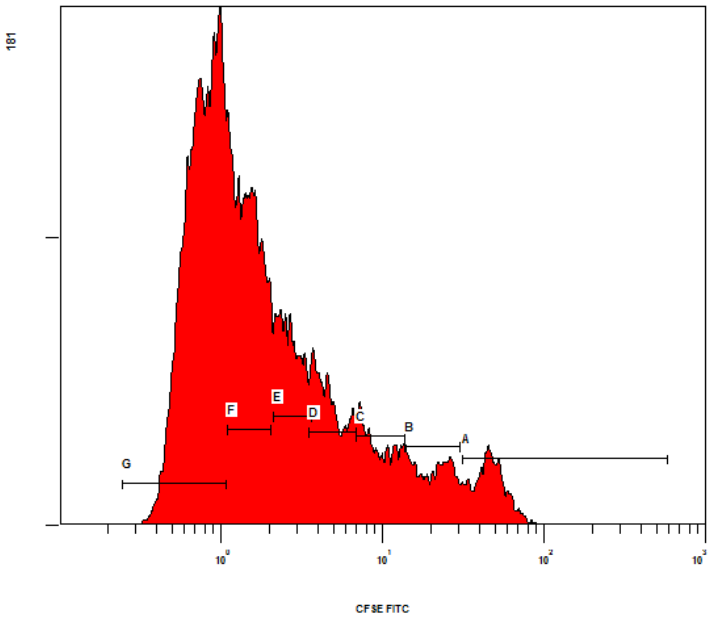


DATA ADQ: 18032019								Calcul index proliferació
CONTROL	**%Mc1**	**%Mc2**	**%Mc3**	**%Mc4**	**%Mc5**	**%Mc6**	**%Mc7**	**Calcul index proliferació**
control sin estimulo	93	2	2	1	1	1	0	=(1*B3+2*C3+4*D3+8*E3+16*F3+32*G3+64*H3)
PMA+IO	6	15	25	30	15	6	3	1000
PHA	6	11	14	20	26	16	6	1556
OKT3 CD4	55	17	6	4	3	5	9	929
OKT3 + IL2 CD4	54	18	7	4	3	5	9	934
OKT3 CD8	35	19	11	8	8	7	13	1365
OKT3 + IL2 CD8	33	20	11	8	7	6	15	1445
PWM	42	9	10	7	11	12	11	1420
Con A CD4	15	7	6	16	19	23	14	2117
Con A CD8	51	3	5	8	12	14	7	1229
131215792	**%Mc1**	**%Mc2**	**%Mc3**	**%Mc4**	**%Mc5**	**%Mc6**	**%Mc7**	**Calcul index proliferació**
control sin estimulo	96	1	1	0	0	0	0	102
PMA+IO	25	21	15	17	10	5	7	1031
PHA	29	15	14	13	12	7	8	1147
OKT3 CD4	32	47	14	3	2	2	2	430
OKT3 + IL2 CD4	60	31	4	1	1	1	2	322
OKT3 CD8	38	48	8	4	1	1	1	310
OKT3 + IL2 CD8	58	37	4	1	0	0	0	156
PWM	64	6	6	5	8	7	6	876
Con A CD4	45	14	11	15	8	6	1	621
Con A CD8	92	3	2	1	1	0	0	130

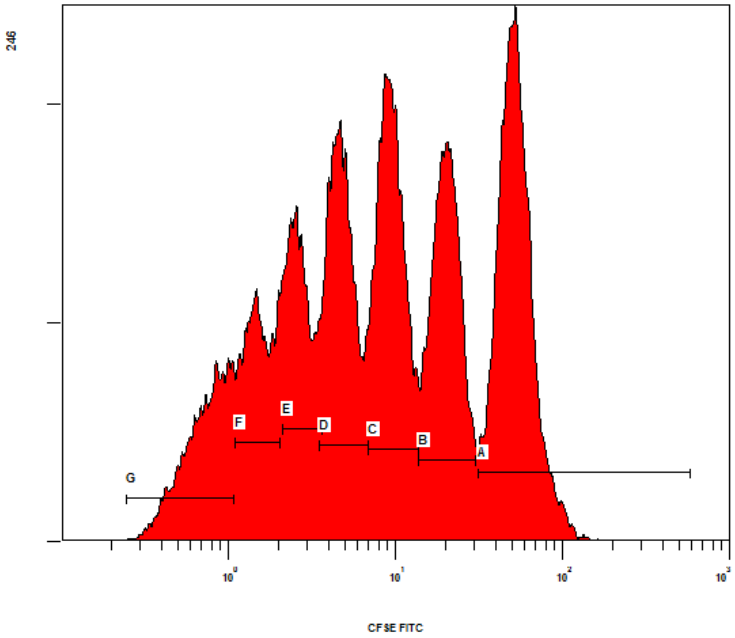
MOSTRA	**SIN EST**	**PMA+IO**	**PHA**	**OKT3 CD4**	**OKT3 + IL2CD4**	**OKT3 CD8**	**OKT3+IL2 CD8**	**PWM**	**ConA CD4**	**ConA CD8**
CONTROL	161	1000	1556	929	934	1365	1445	1420	2117	1229
131215792	102	1031	1147	430	322	310	156	876	621	130



Healthy control



Patient 131216120



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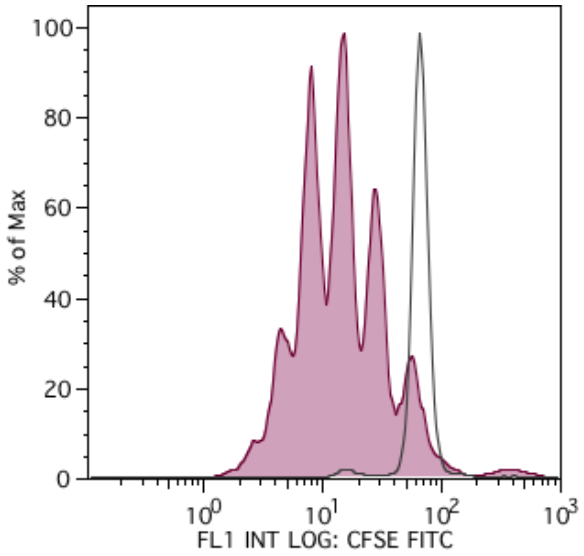
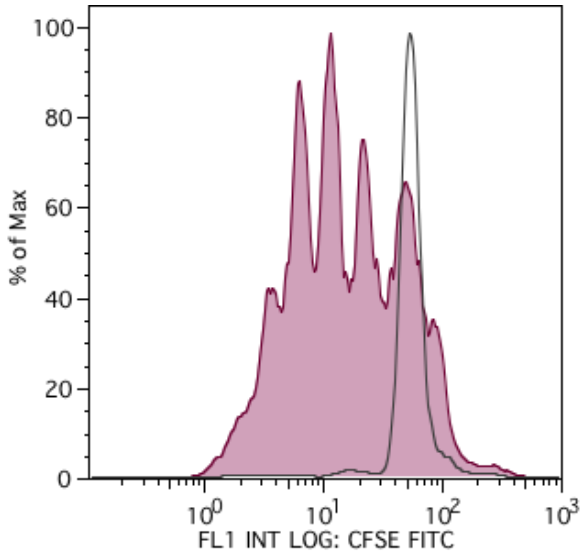
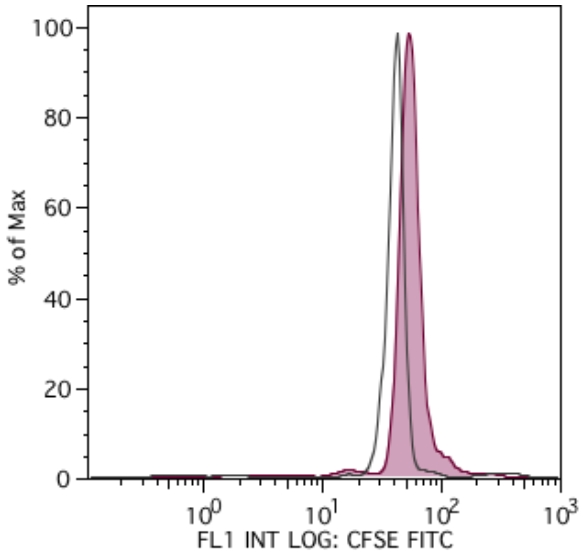
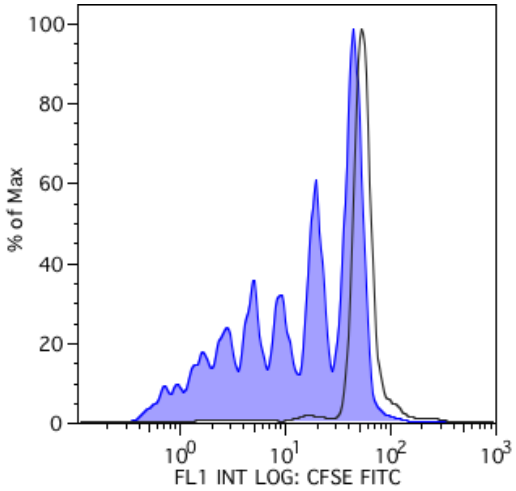
PMA+ionomicine

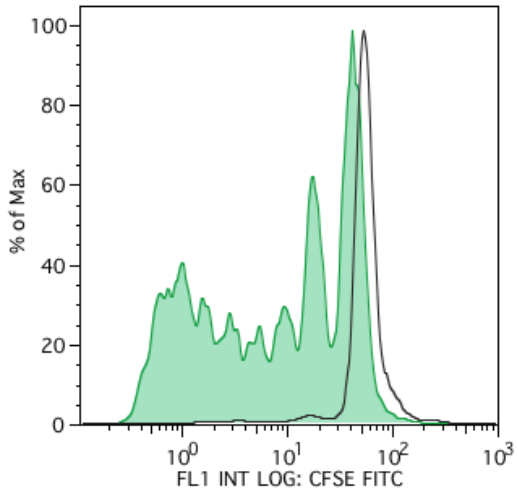






Anti-CD3+IL2





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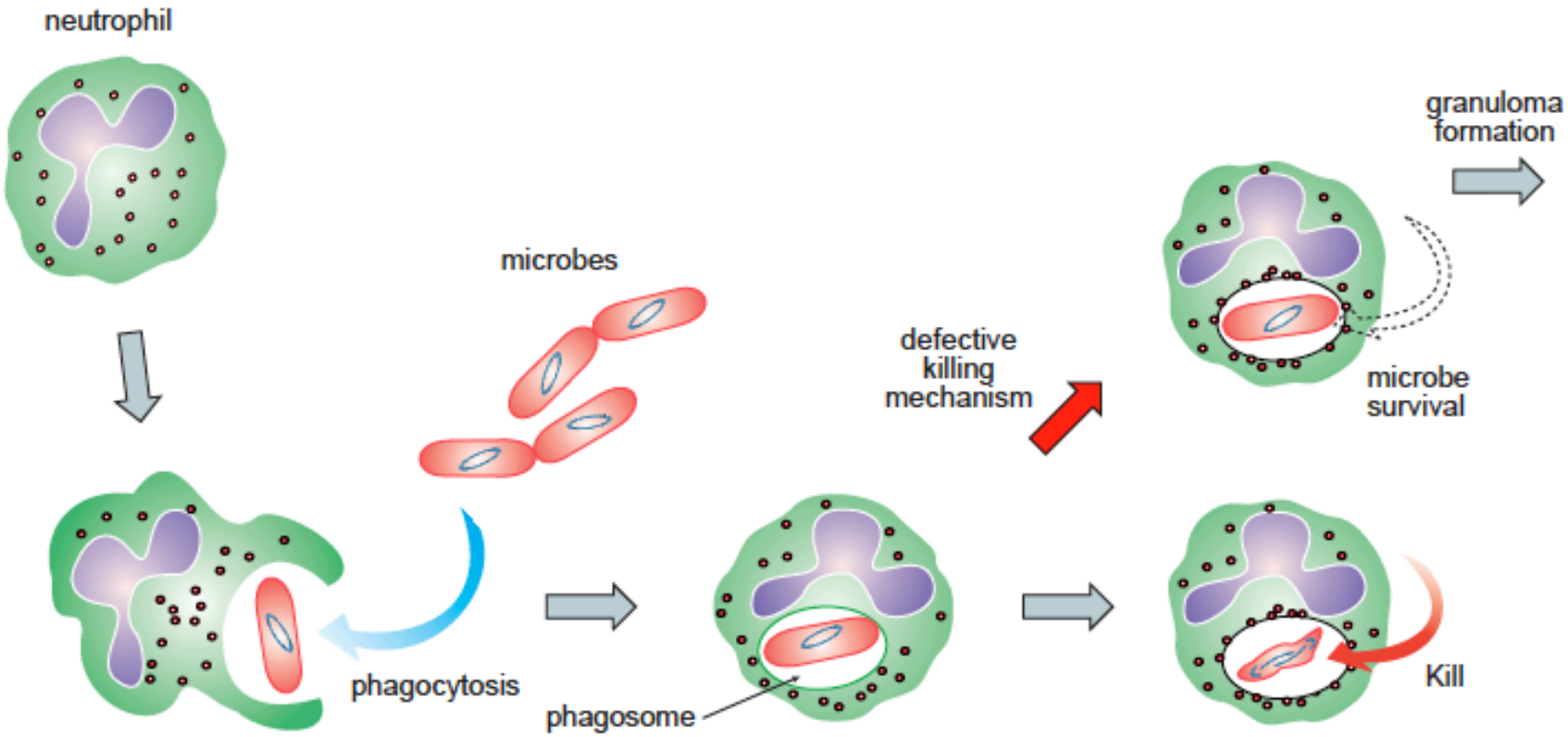
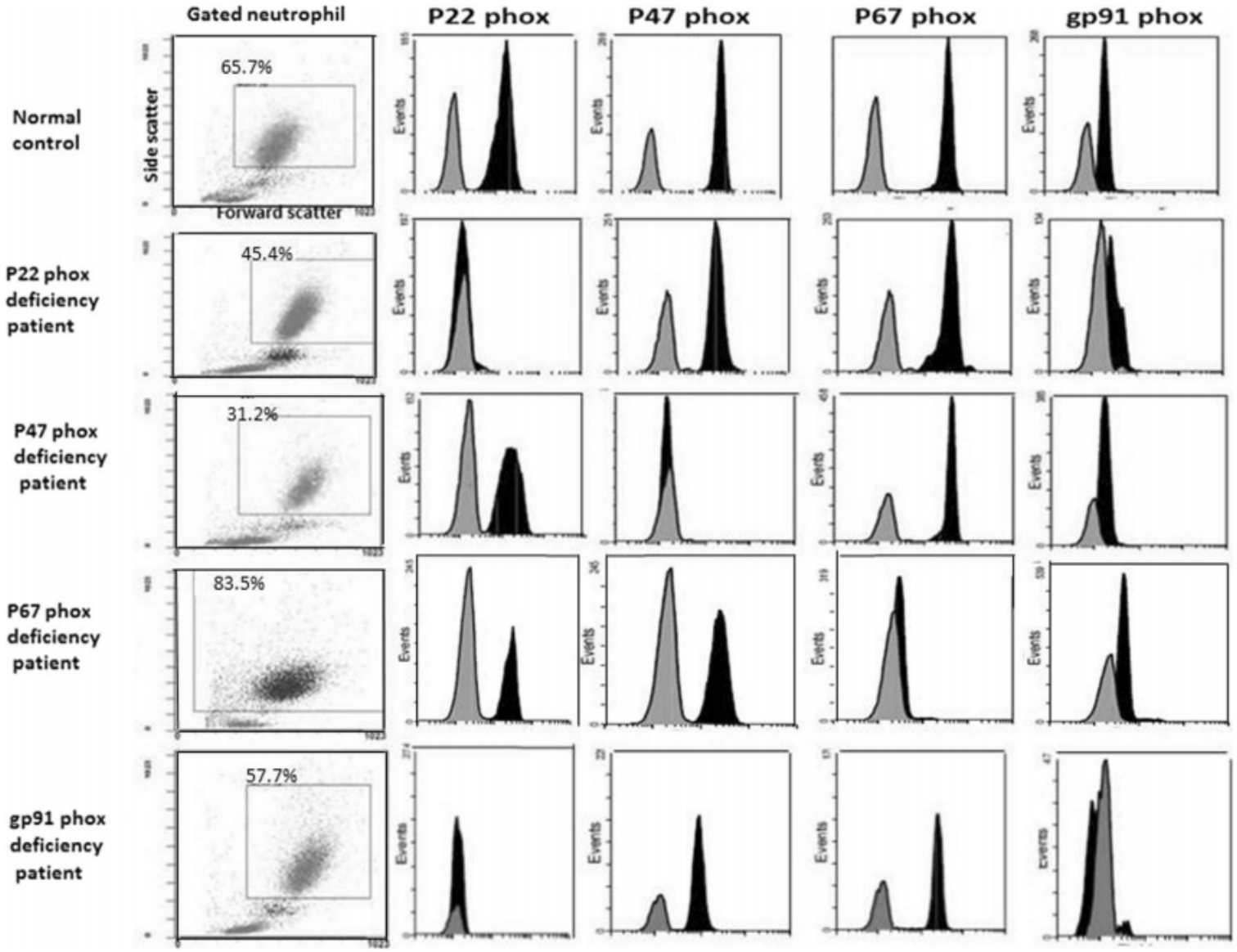
Neutrophil respiratory burst test: Diagnostic of Chronic Granulomatous Disease

In 1967 Baehner and Nathan: leukocytes failed to reduce NBT dye → defective oxidase



Flow cytometry: protein expression



J Clin Immunol. 2016 Aug;36(6):610-8

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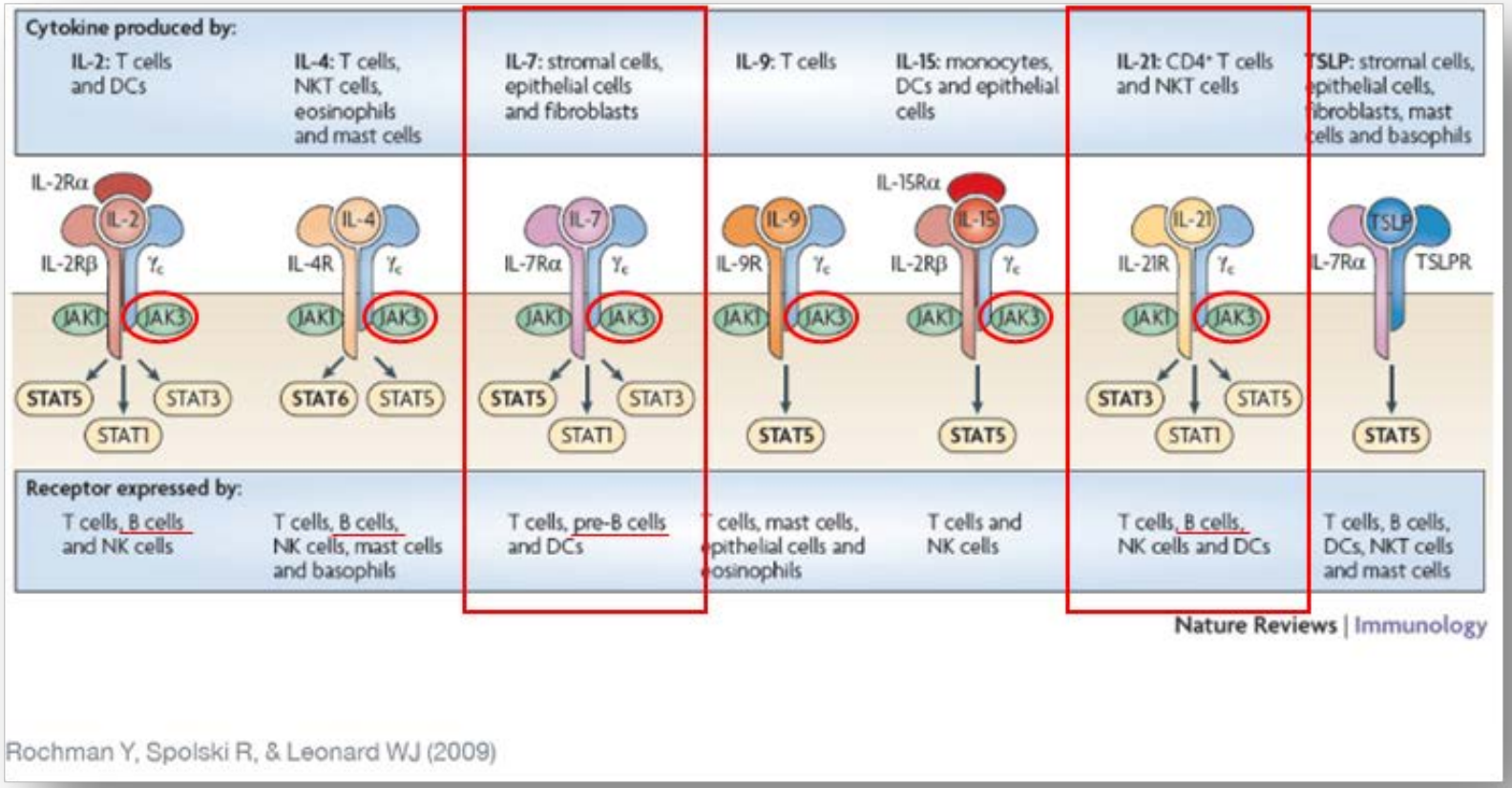
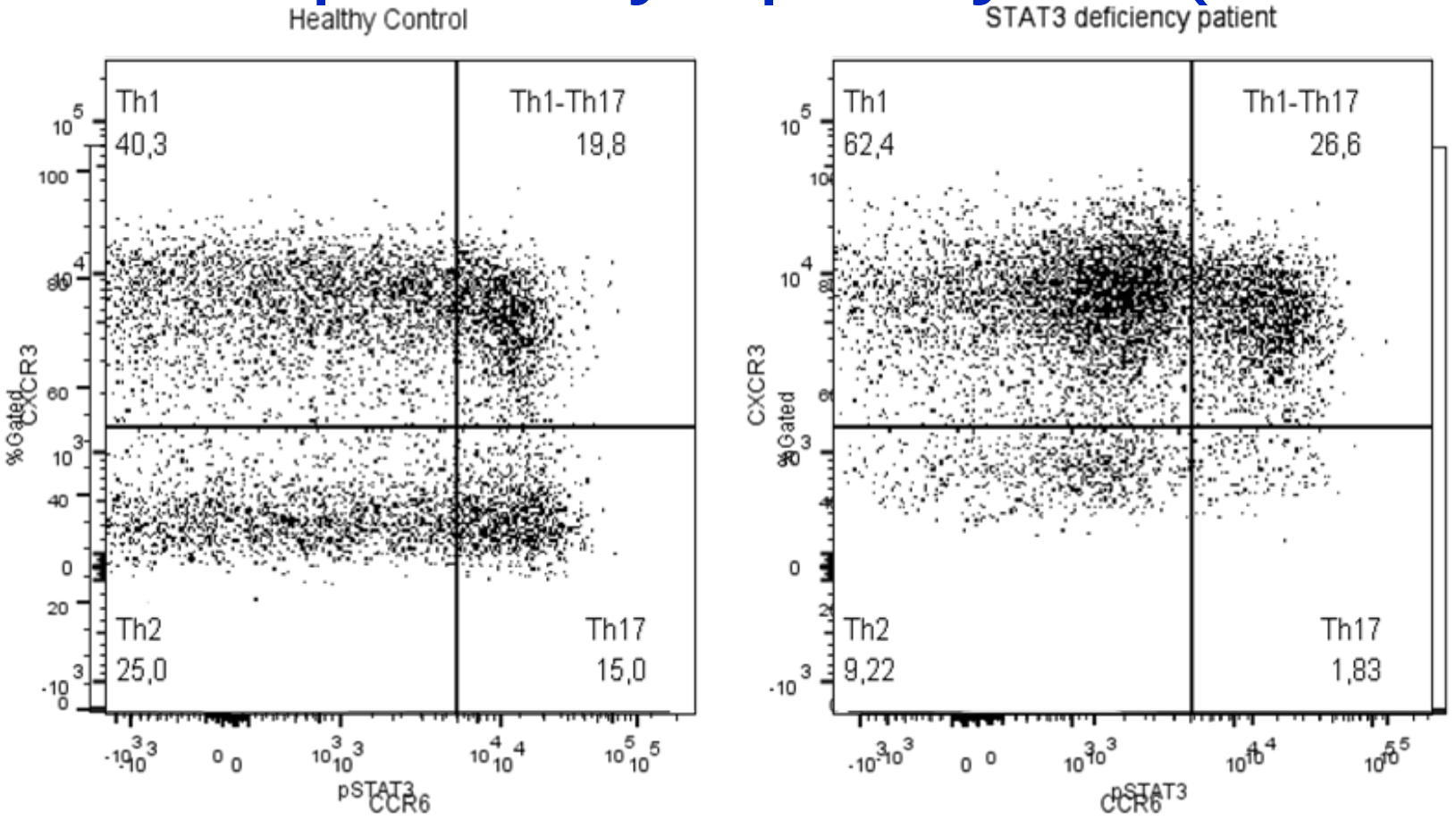
Phosphorylation assays



Rochman Y, Spolski R, & Leonard WJ (2009)

STAT3p in T Lymphocytes (CD3+)



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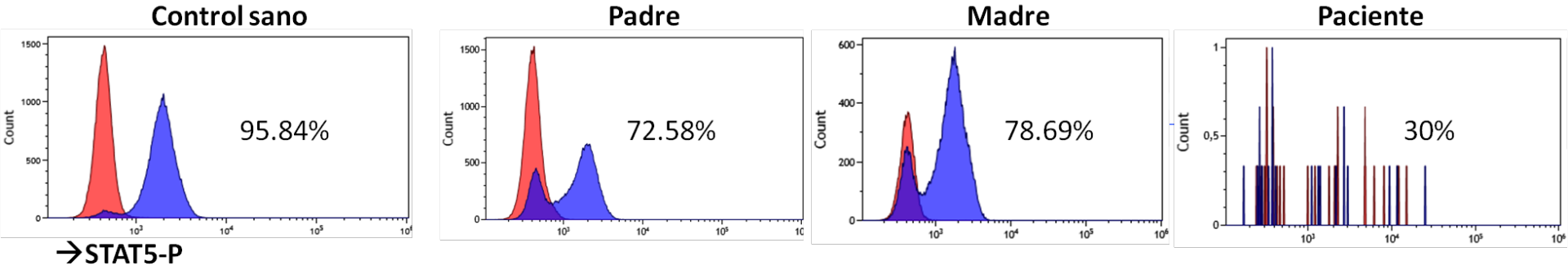
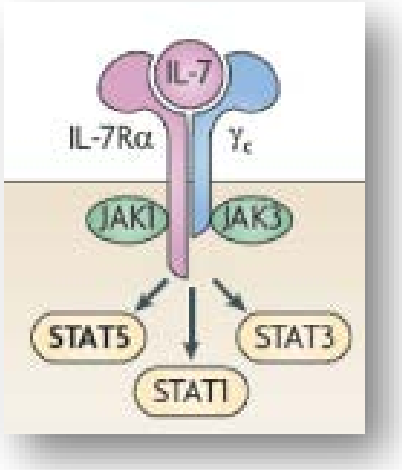
STAT5p in T Lymphocytes (CD3+)





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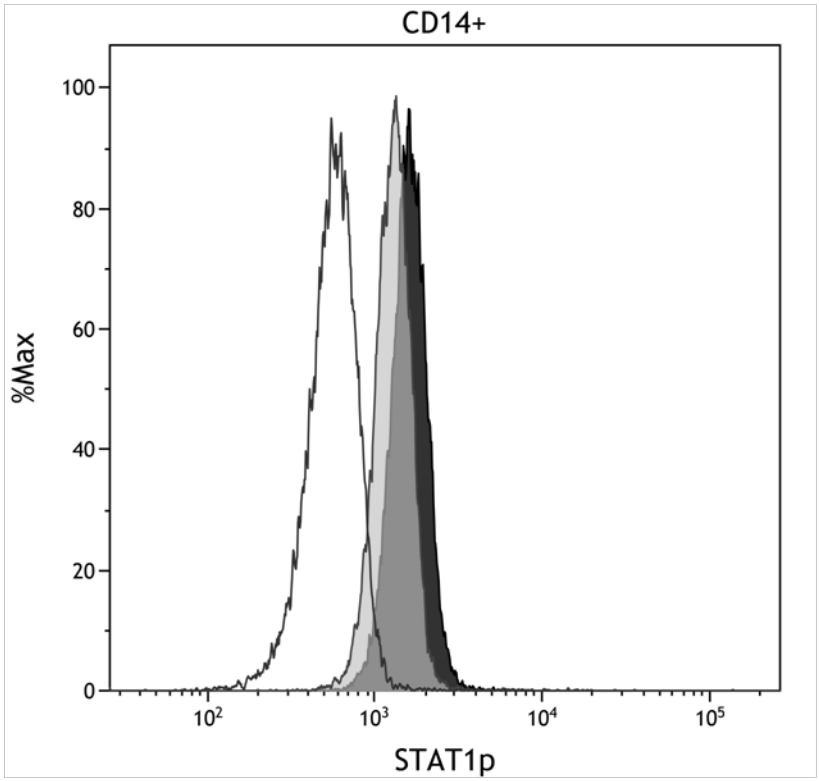
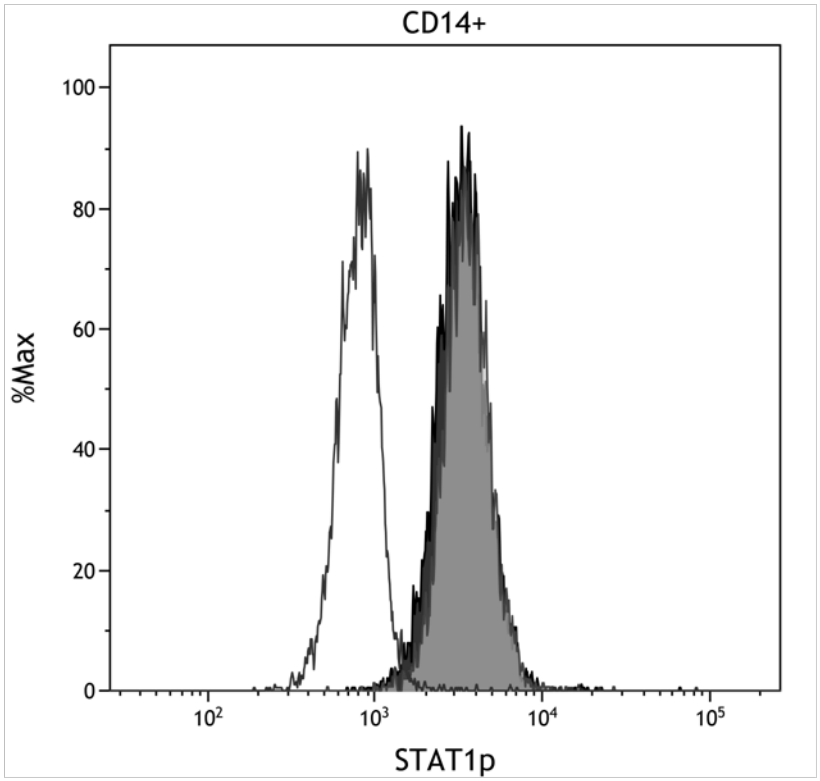
STAT1p in Monocytes (CD14+)





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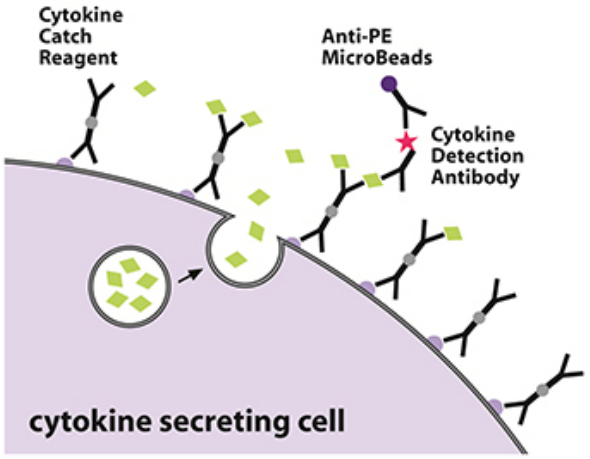
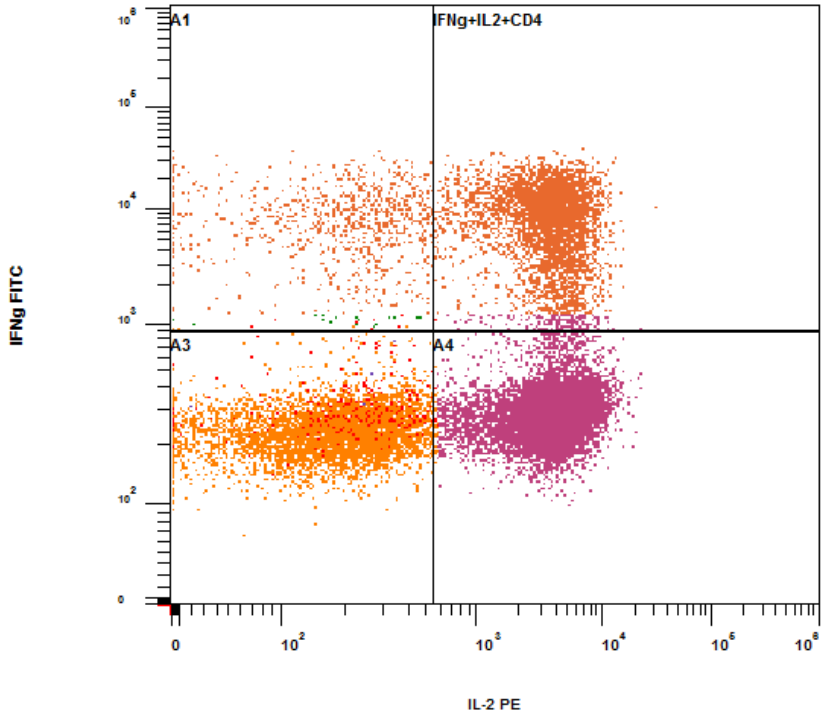
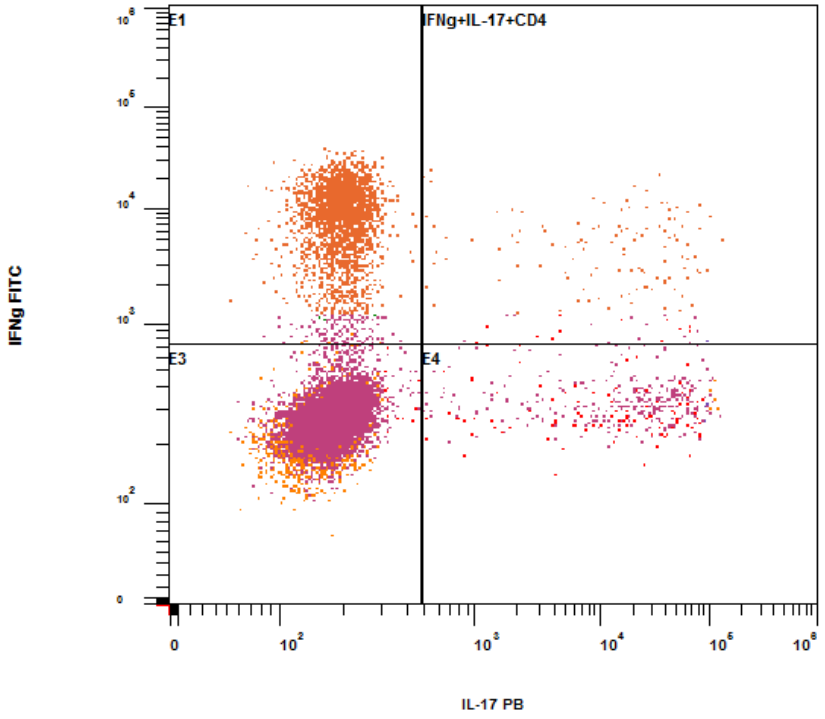


Intracellular cytokines





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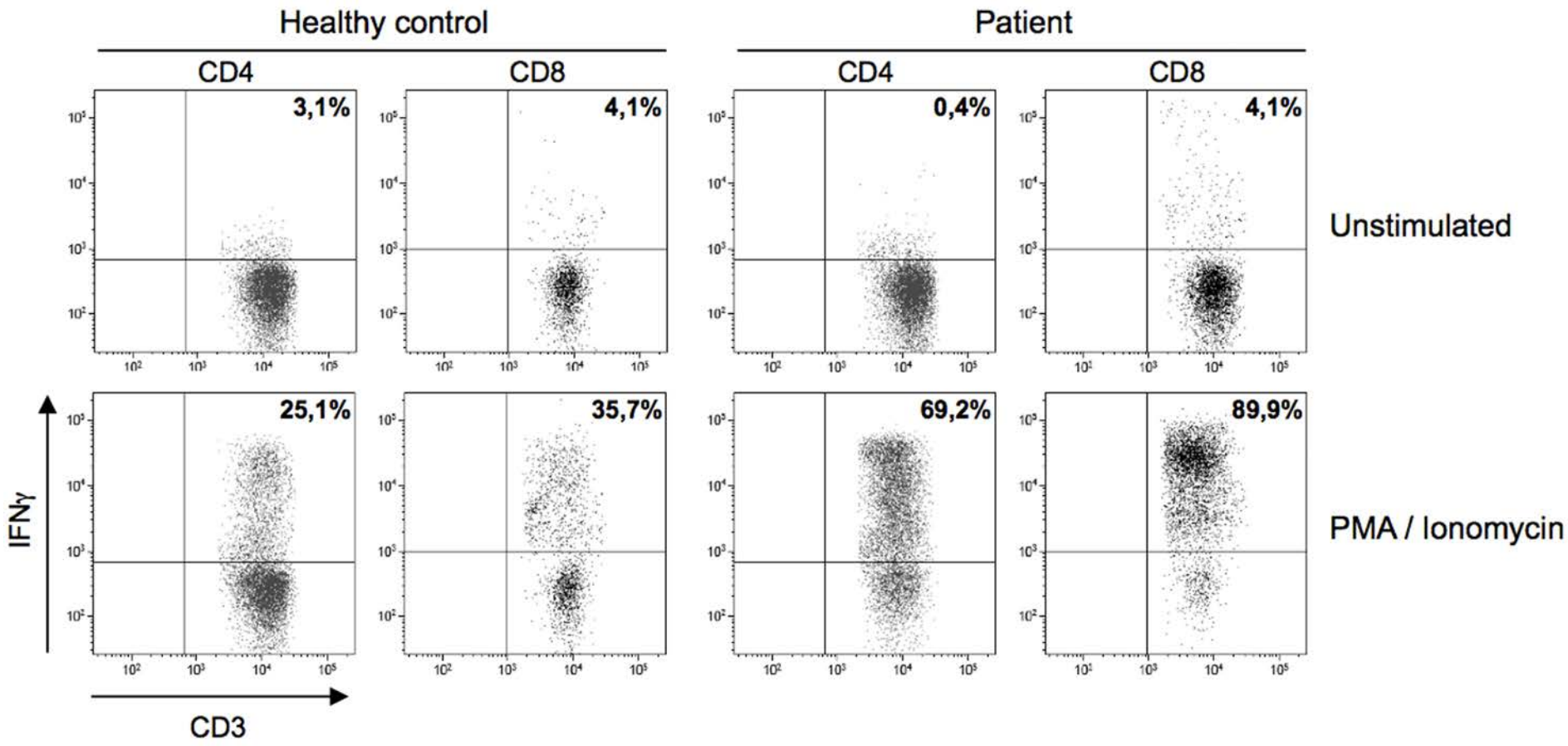
Intracellular cytokines



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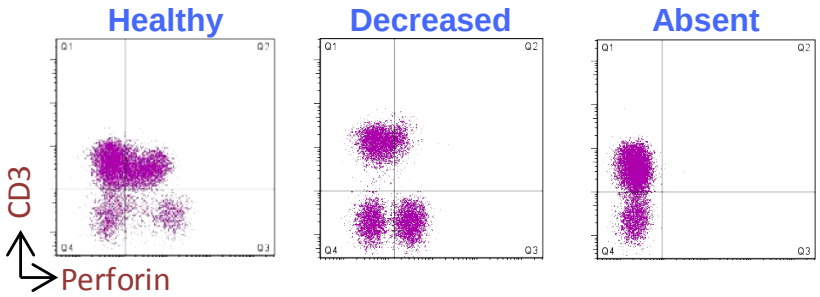
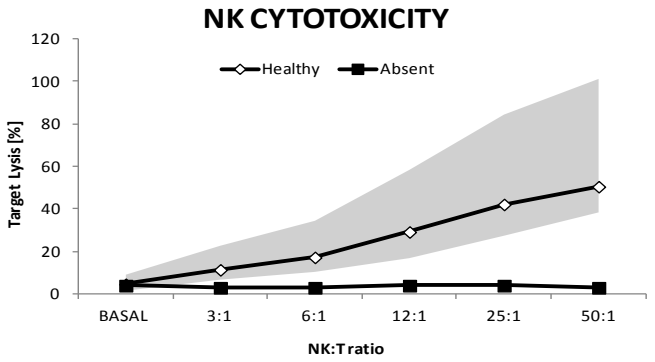
FHL Diagnostic work-up

1. Functional and phenotype tests

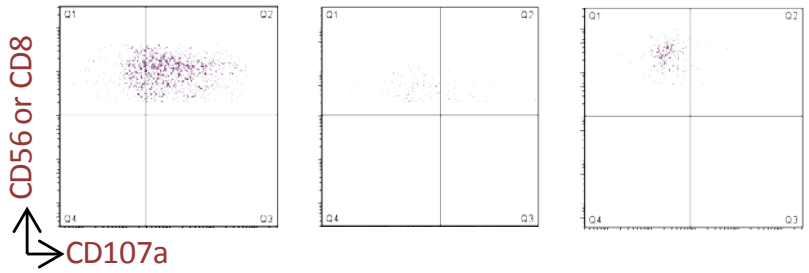
✓Perforin expression



✓Cytotoxic activity



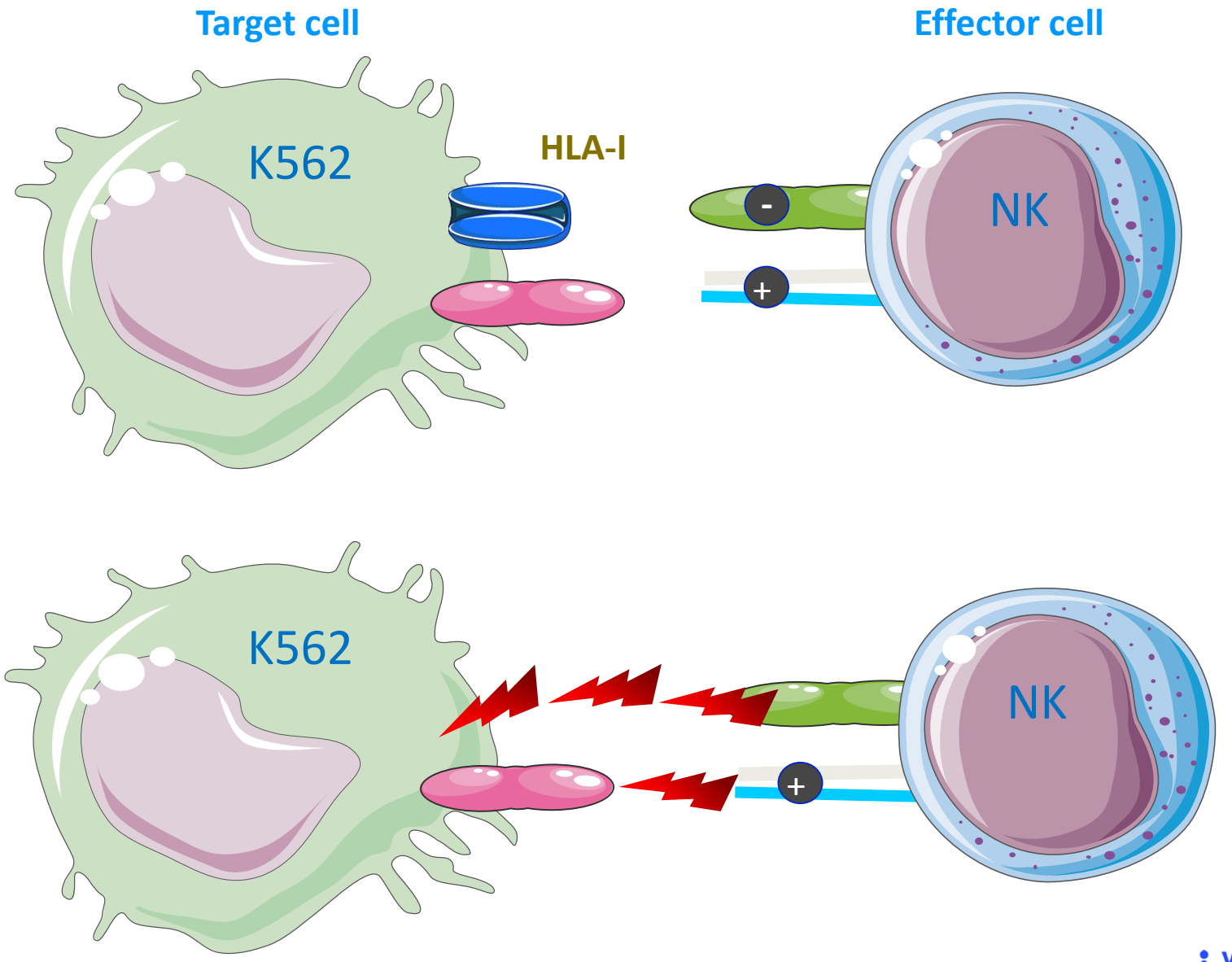
✓Degranulation assay



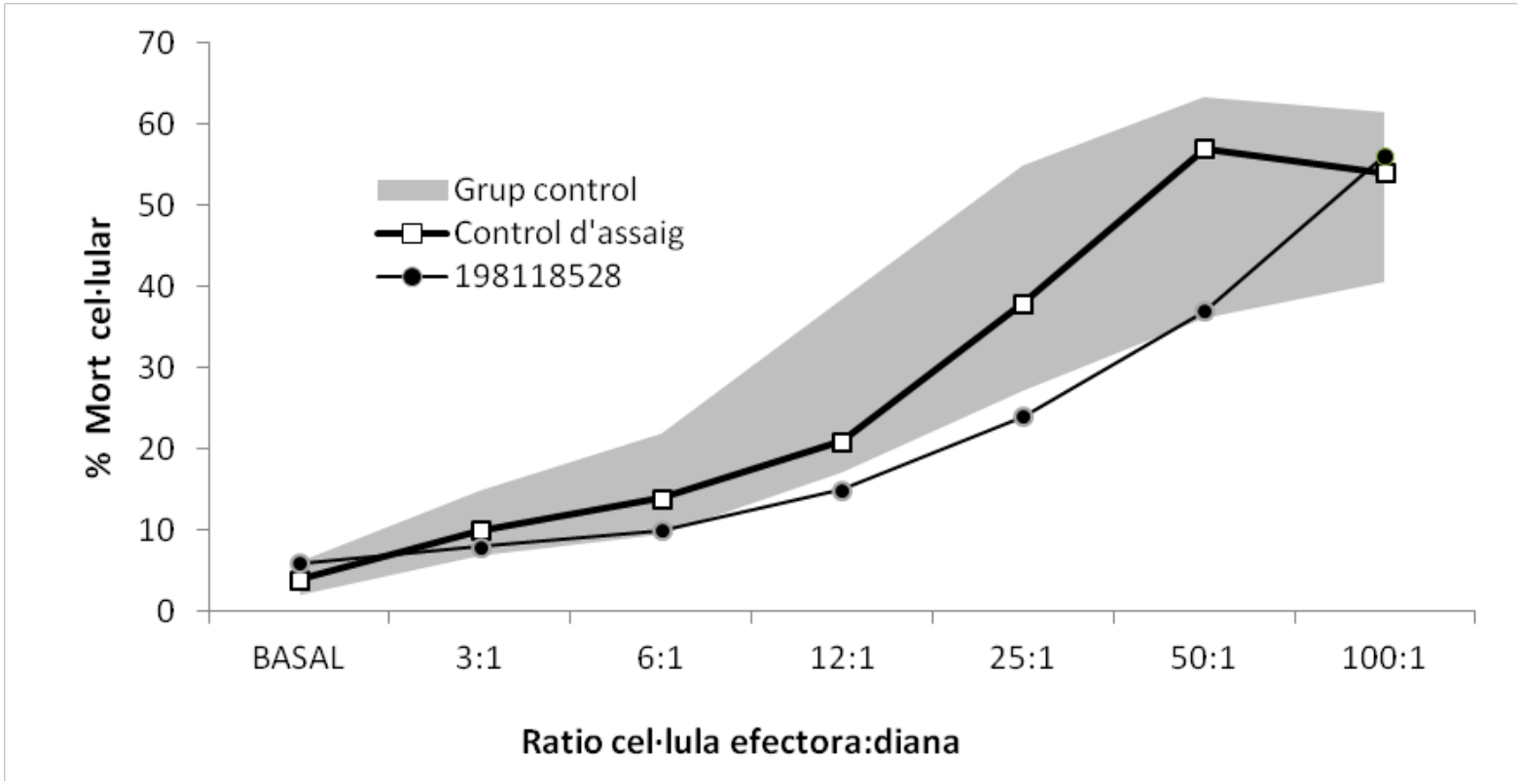
2. Genetic study

- FHL2: Perforin mutations
- FHL3: UNC13D mutations
- FHL4: STX11 mutations
- FHL5: STXBP2 mutations
- Chediak-Higashi: LYST mutations
- Griscellis type 2: Rab27 mutations
- Hermansky-Pudlak type 2: AP3B1 mutations
- XLP: SAP/XIAP mutations

Cytotoxic activity



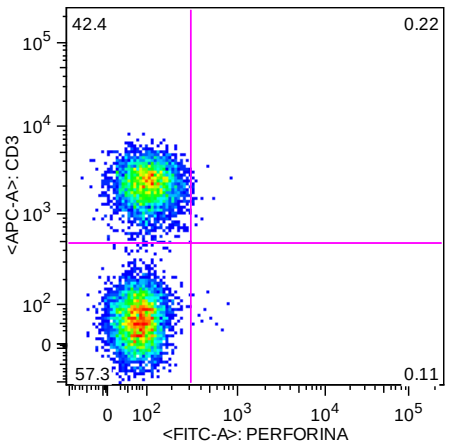
Cytotoxic activity



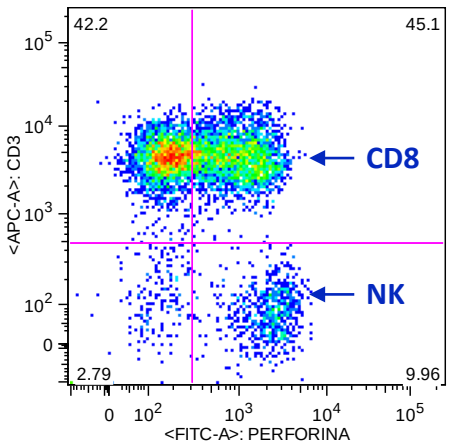
Vall d'Hebron

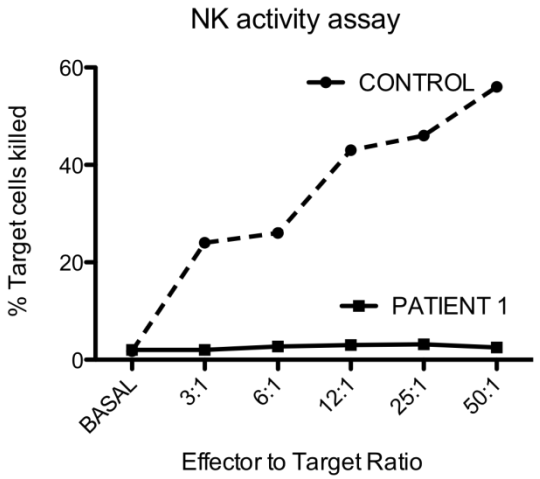
Genetic and functional study of *PRF1* mutations

ISOTYPE CONTROL

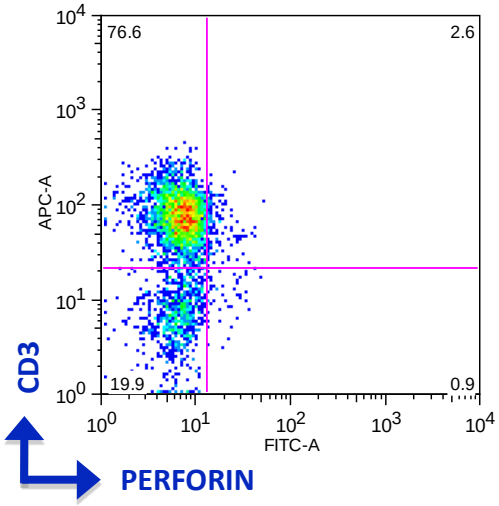


HEALTHY DONOR





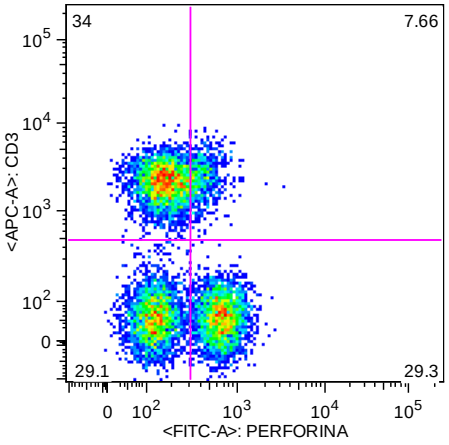
PACIENT 1



Mut *PRF1*

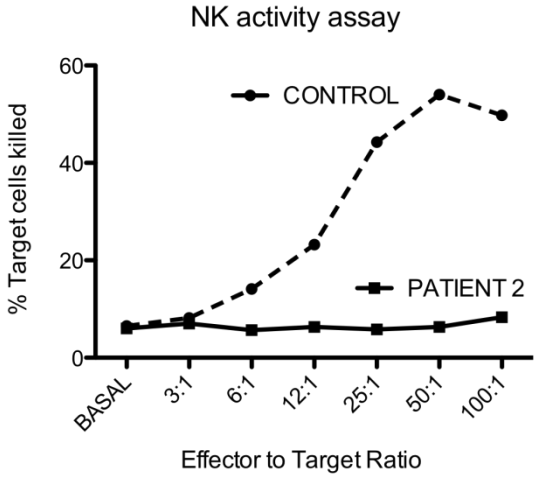
p.Gly477fs →X479

PACIENT 2



Mut *PRF1*

p.Pro459Leu



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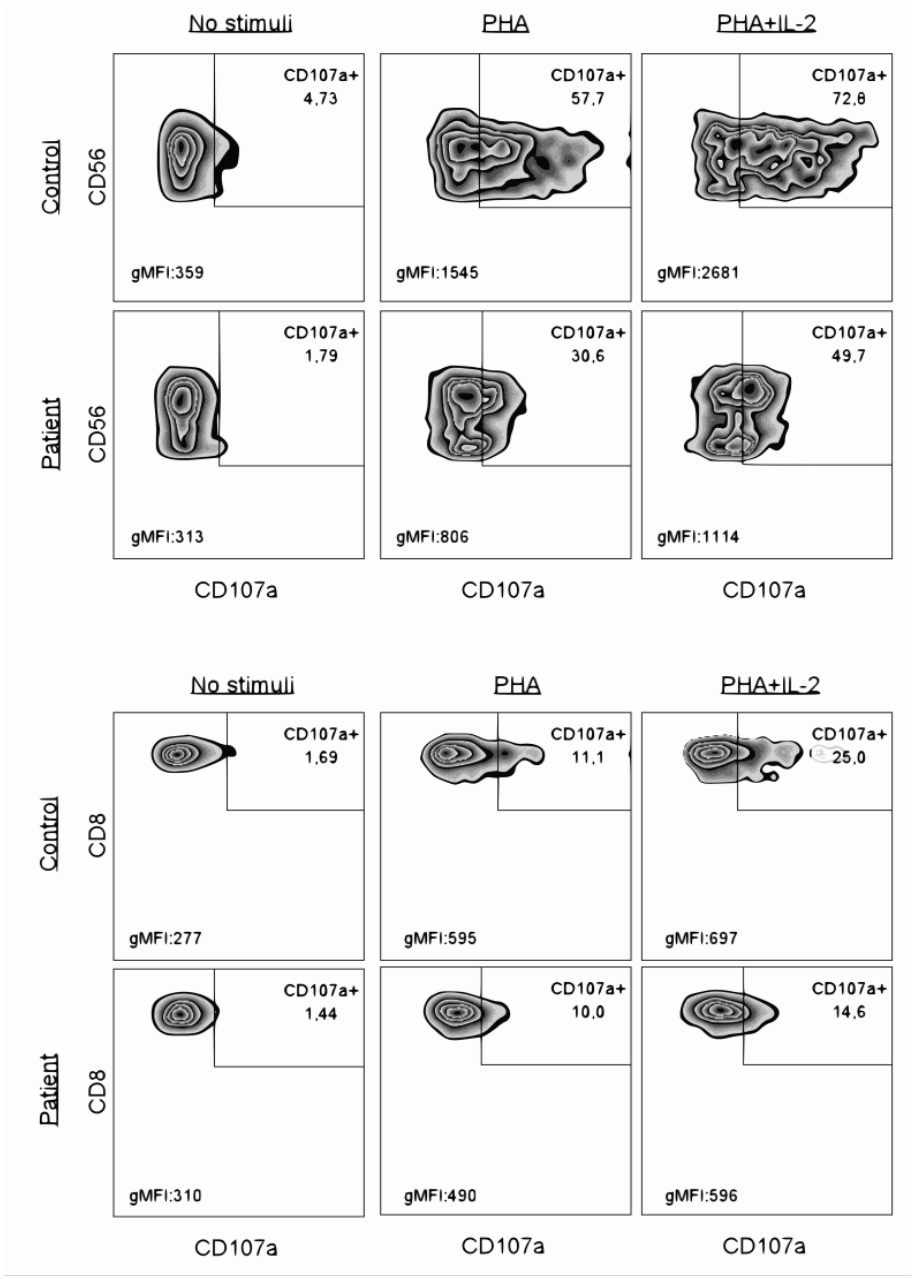

Degranulation assay

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Plan your experiment

Servei d'Immunologia

- Dr. Manuel Hernandez
- Dr. Ricardo Pujol
- Sandra Salgado
- Sara Briongos
- Karen Mendoza
- Marina García Prat
- Clara Franco
- Laura Viñas
- Dani Álvarez
- Gemma Vila

Vall d'Hebron
Barcelona Hospital Campus

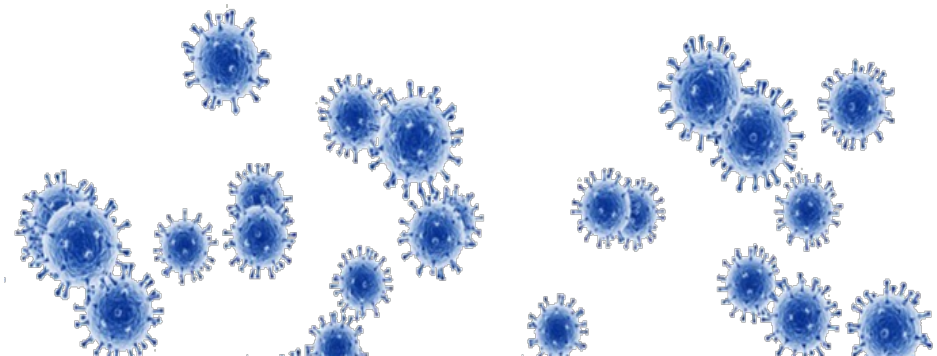
Unión Europea
Fondo Europeo de Desarrollo Regional
"Una manera de hacer Europa"

Vall d'Hebron
Institut de Recerca

Identification by RNA/Flow assay subpopulations of CD4 lymphocytes that express HIV-1 RNA

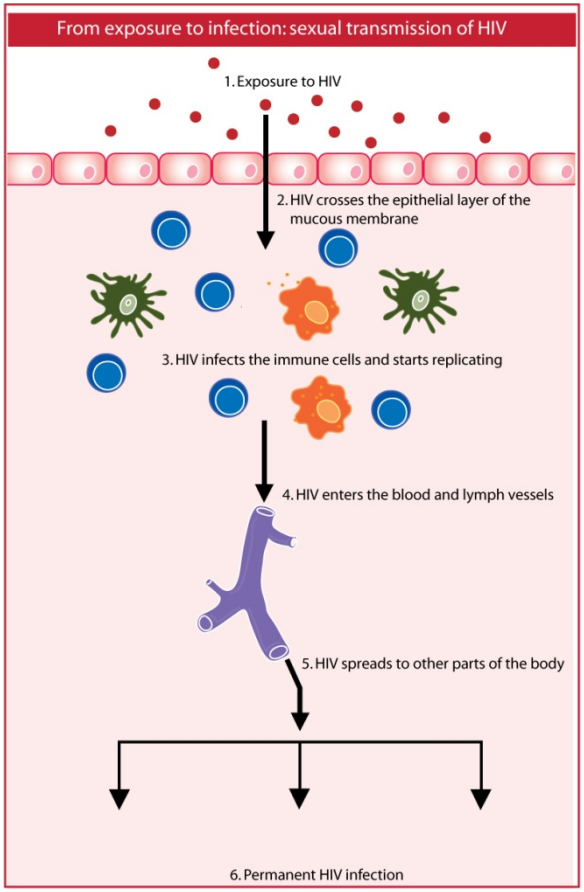
Carla Serra Peinado, PhD

Department of Infectious Diseases, Hospital Universitari Vall d'Hebrón, Institut de Recerca (VHIR)
Universitat Autònoma de Barcelona

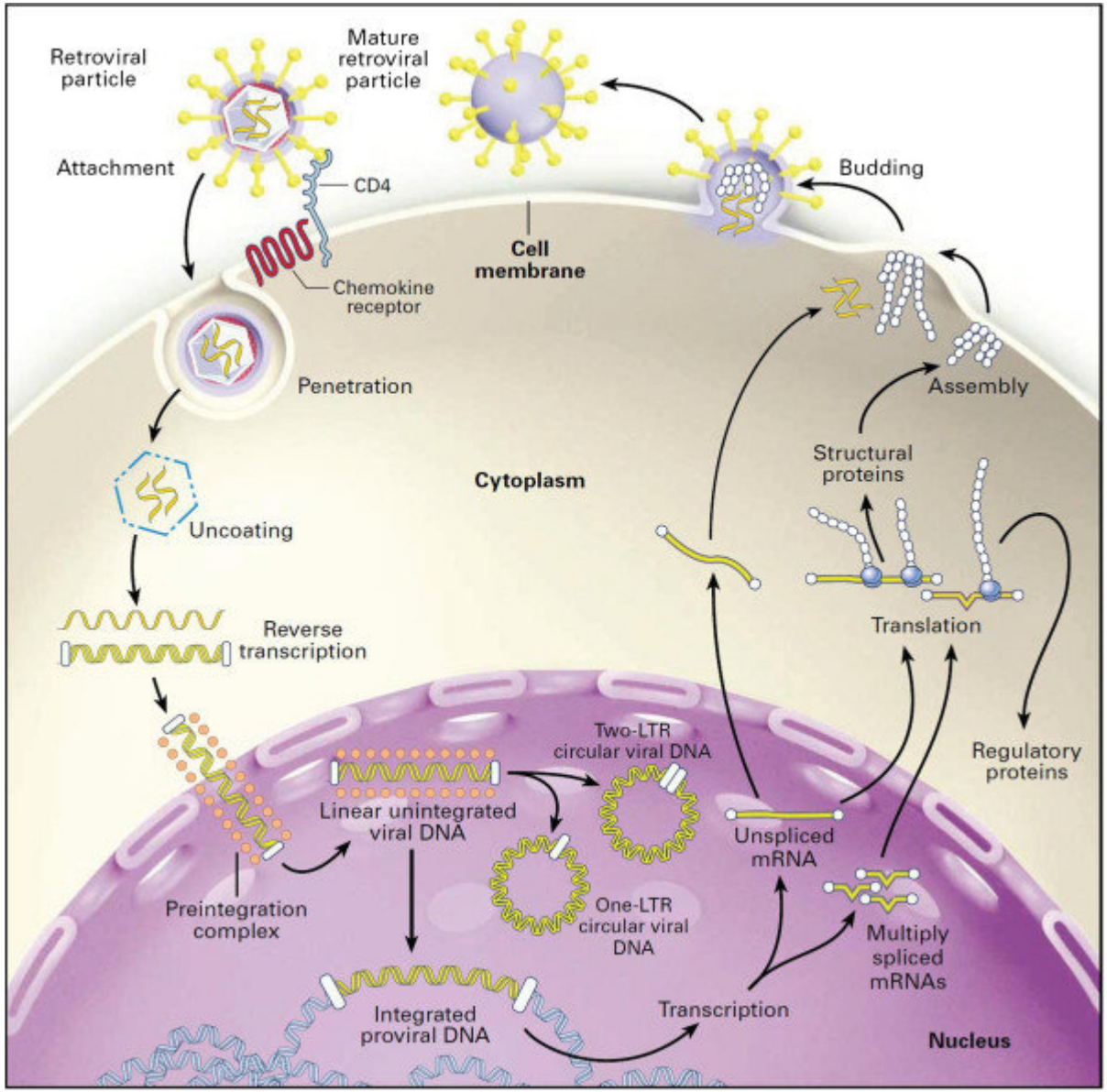


HIV infection

HIV entrance


From exposure to infection: sexual transmission of HIV
1. Exposure to HIV
2. HIV crosses the epithelial layer of the mucous membrane
3. HIV infects the immune cells and starts replicating
4. HIV enters the blood and lymph vessels
5. HIV spreads to other parts of the body
6. Permanent HIV infection

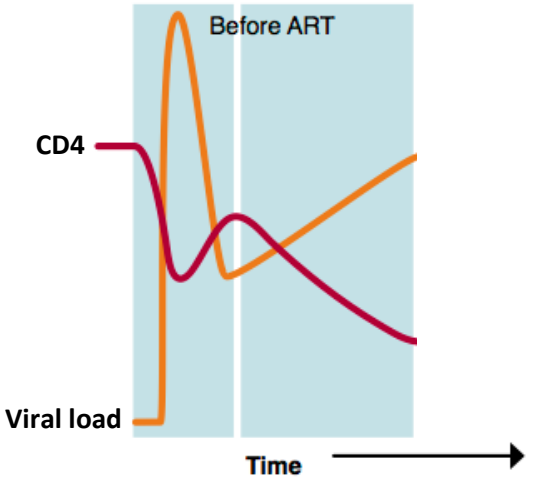
HIV cycle



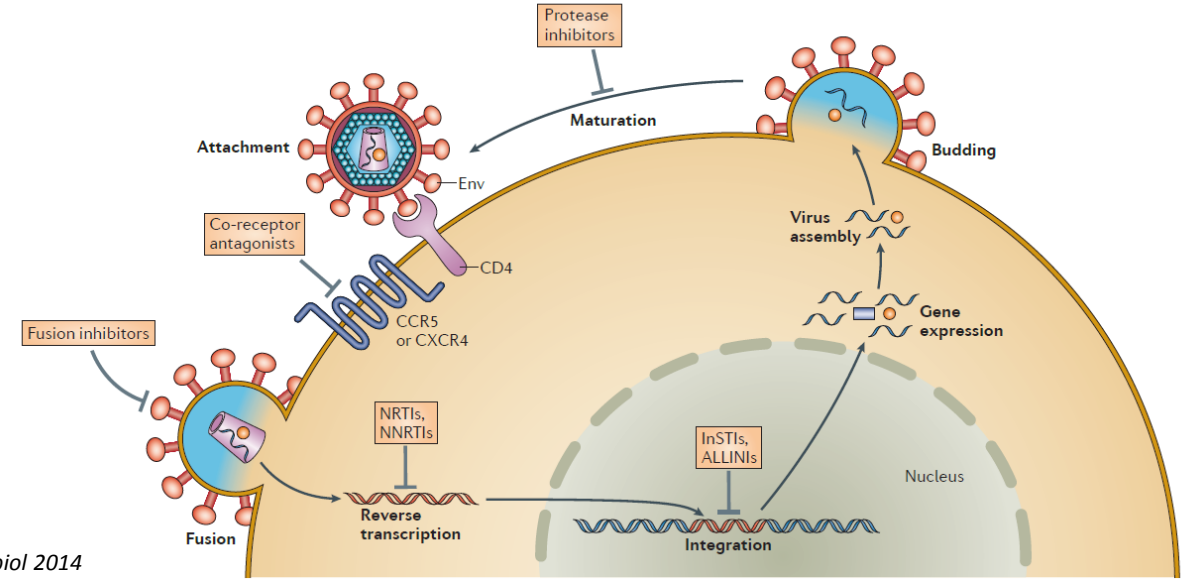
Pasternak AO et al. Retrovirology 2013

HIV infection

Viral load and CD4 T cells:



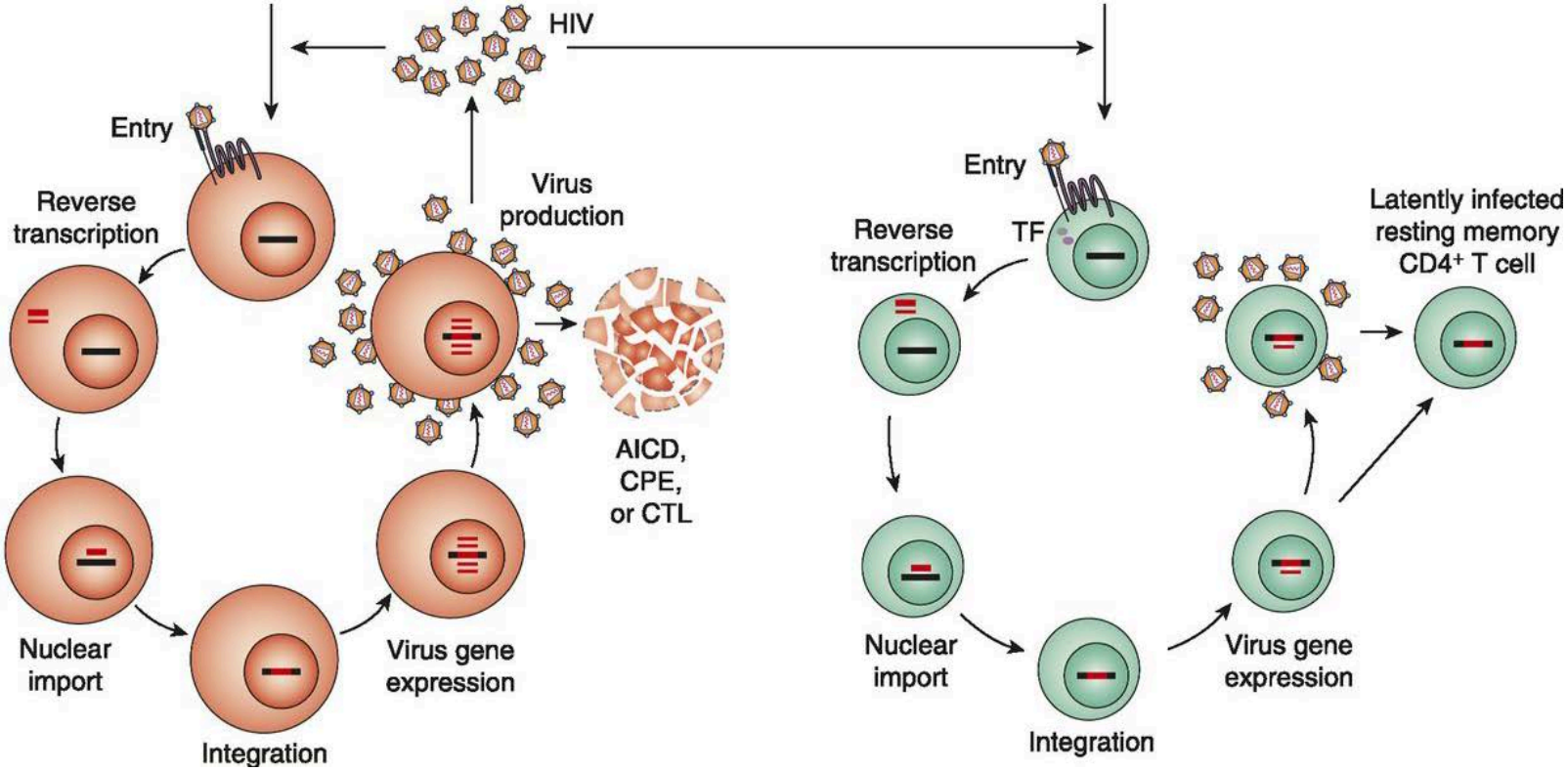
Antiretroviral drug treatment (ART):



Laskey SB et al. Nat Rev Microbiol 2014

HIV reservoir generation

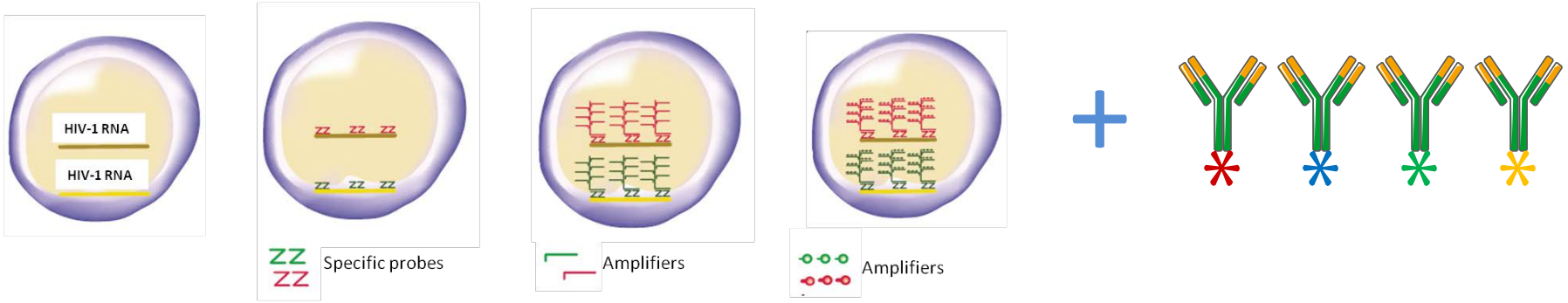
Establishment of HIV latency:



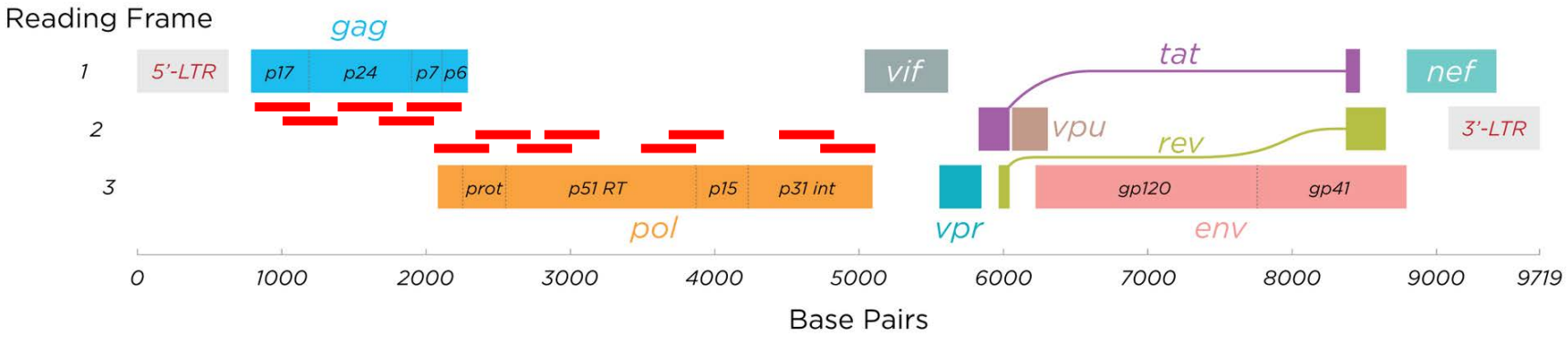
Murray AJ et al. J Immunol 2016

RNA/Flow assay for detection of HIV-1 RNA

RNA/Flow assay:

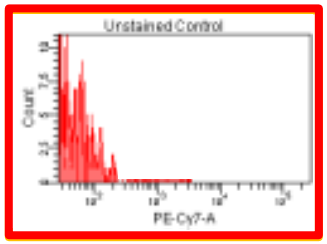


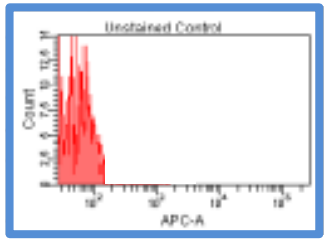
Probes:

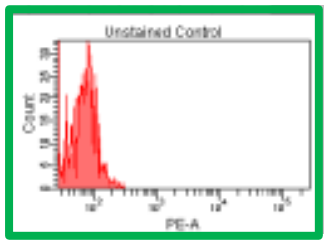


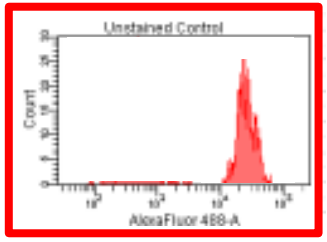
A high-sensitivity target-specific set of 50 individual probes targeting the HIV RNA Gag-Pol sequence
(bases 1165 to 4402 of the HXB2 consensus genome)

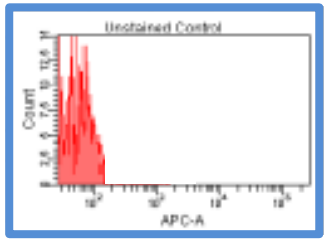
Compensation

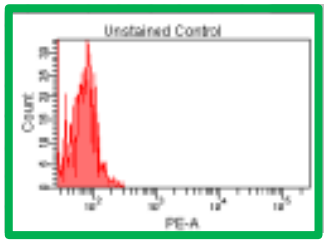


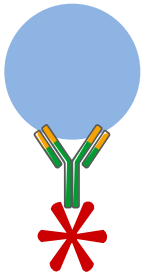


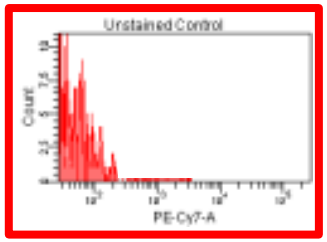


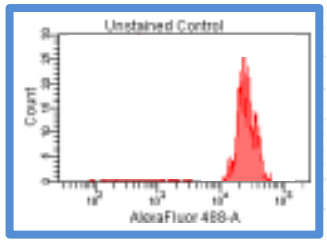


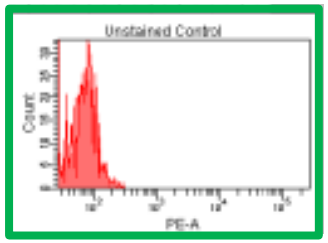


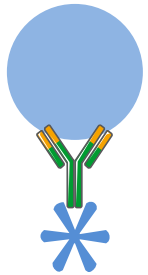


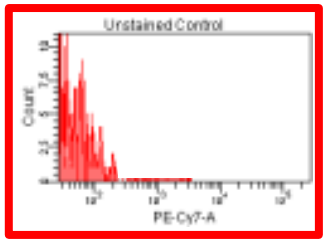


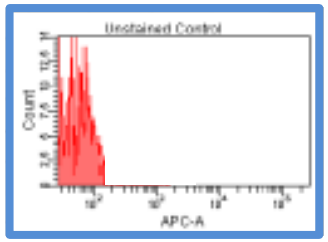


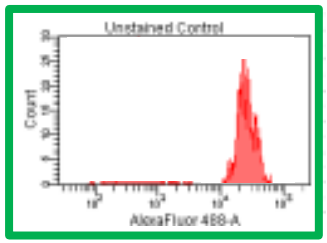


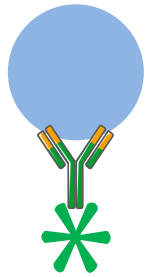




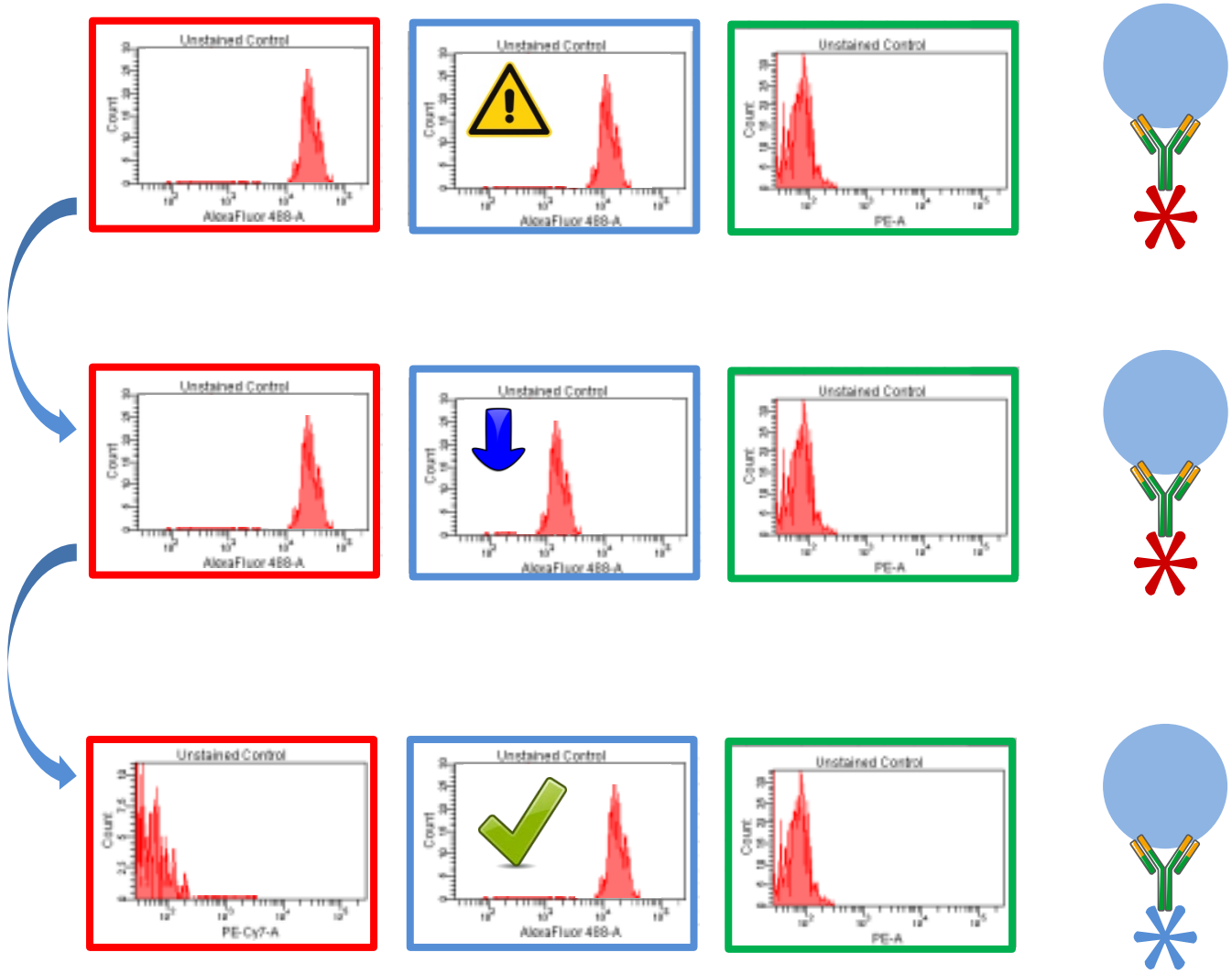








Compensation



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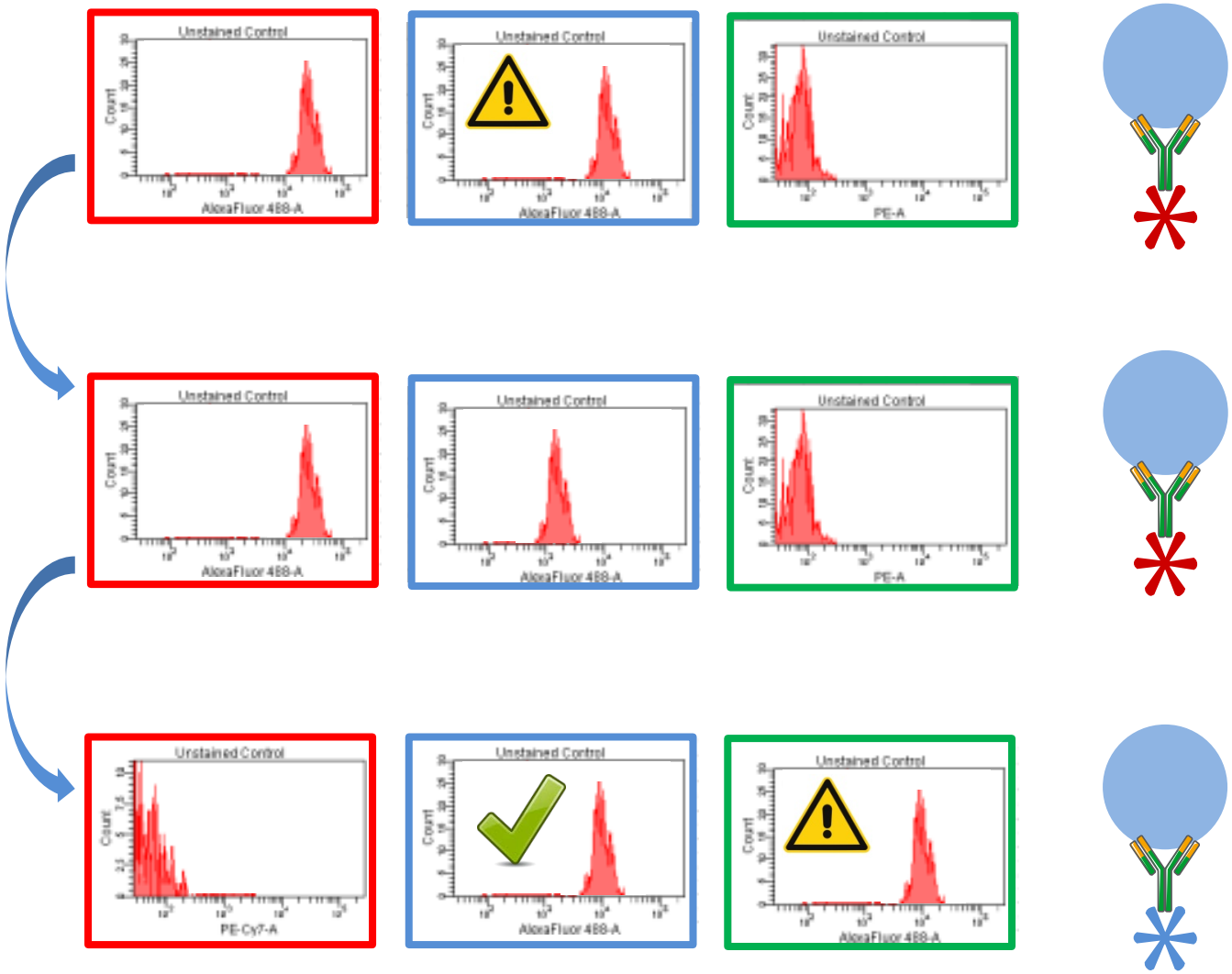

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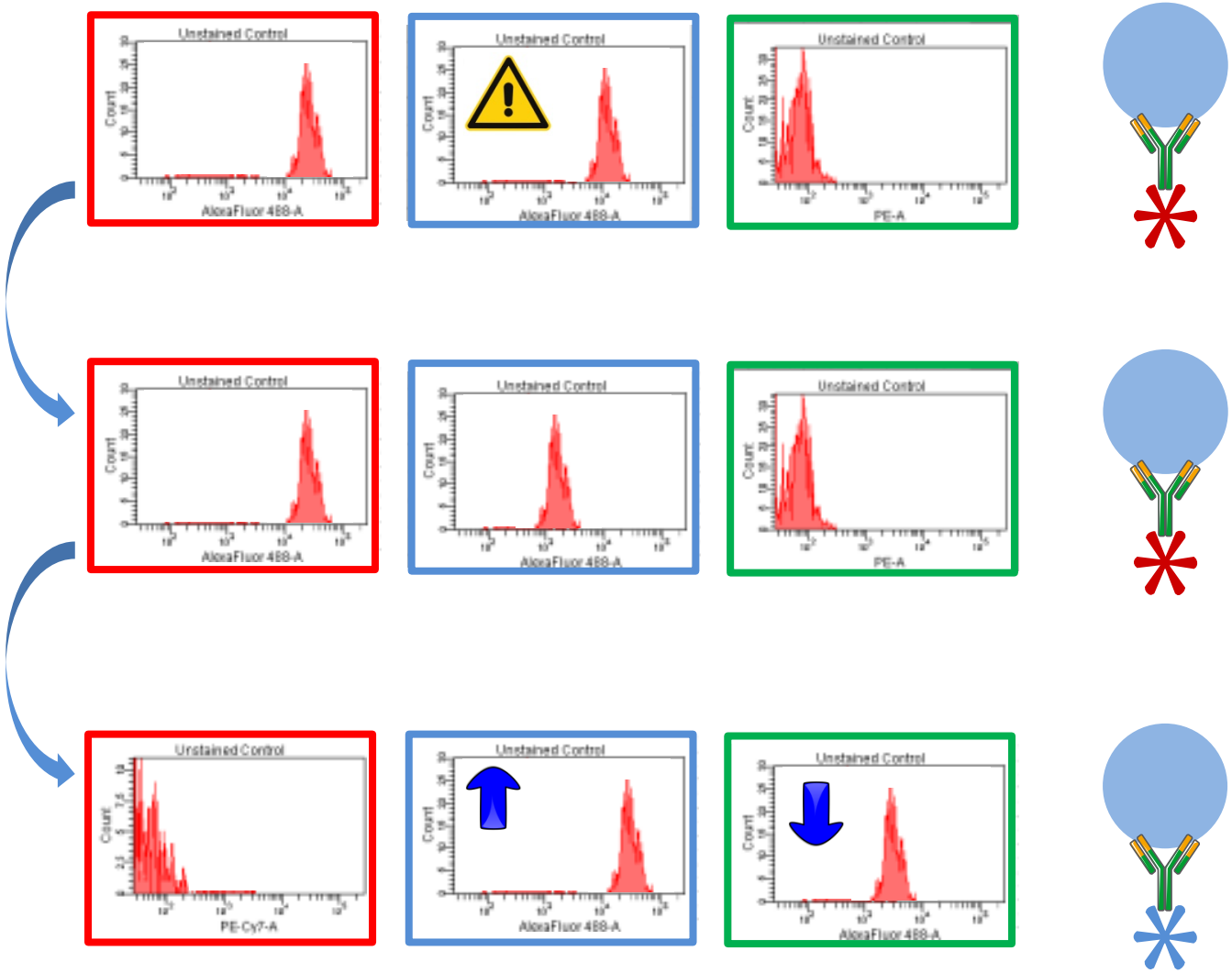

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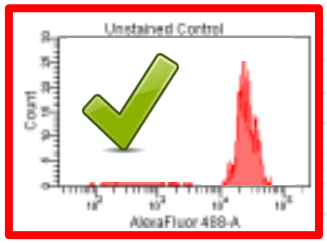
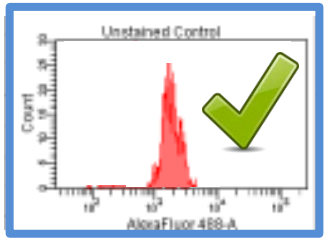
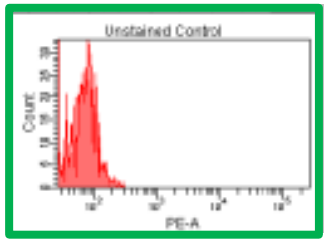
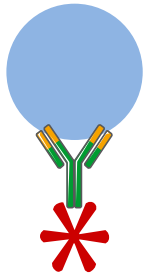
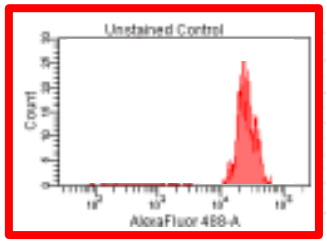
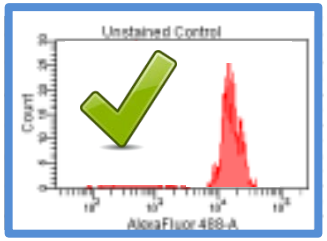


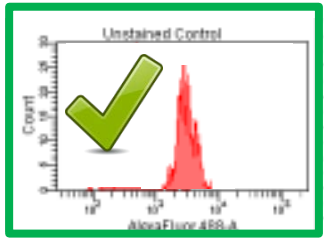


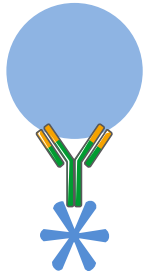


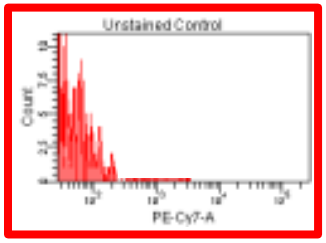


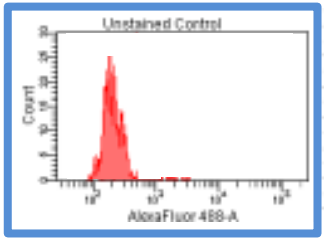


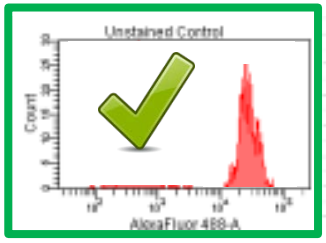


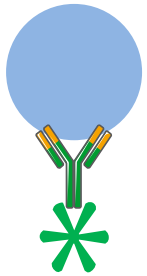






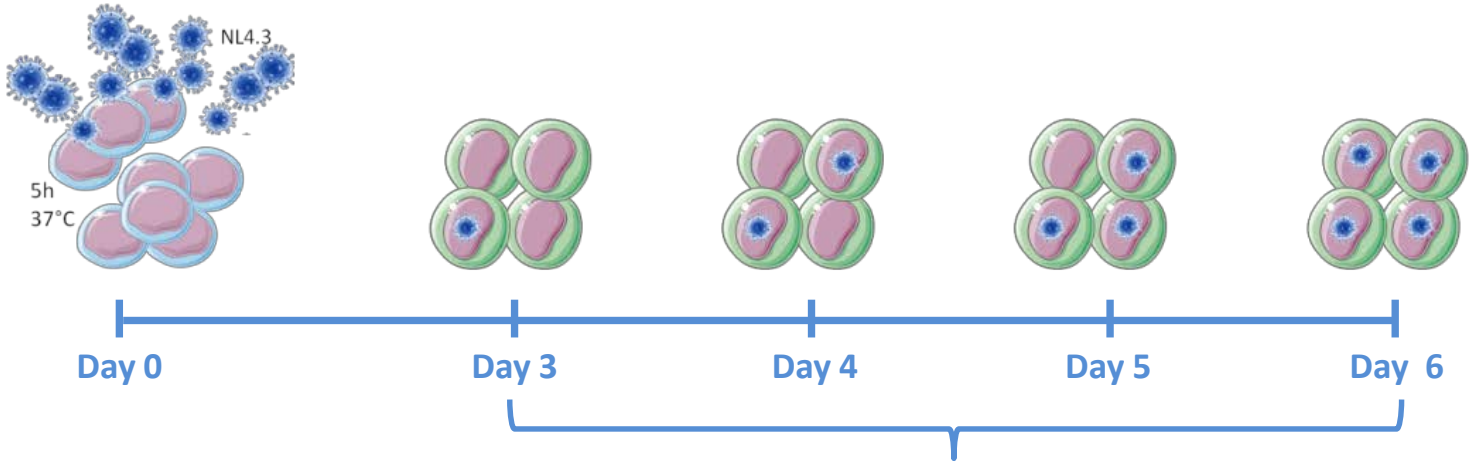


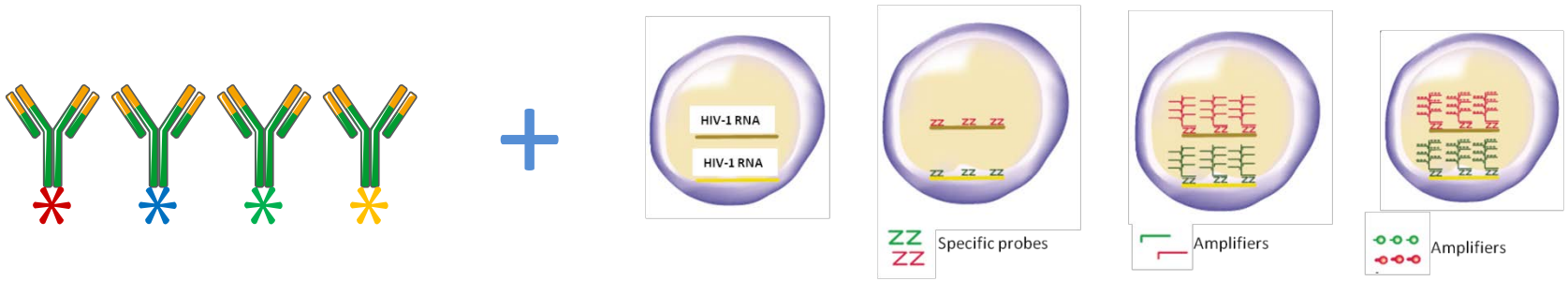




Detection of HIV-1 RNA in exvivo infected PBMCs

✓ **Experimental procedure:**



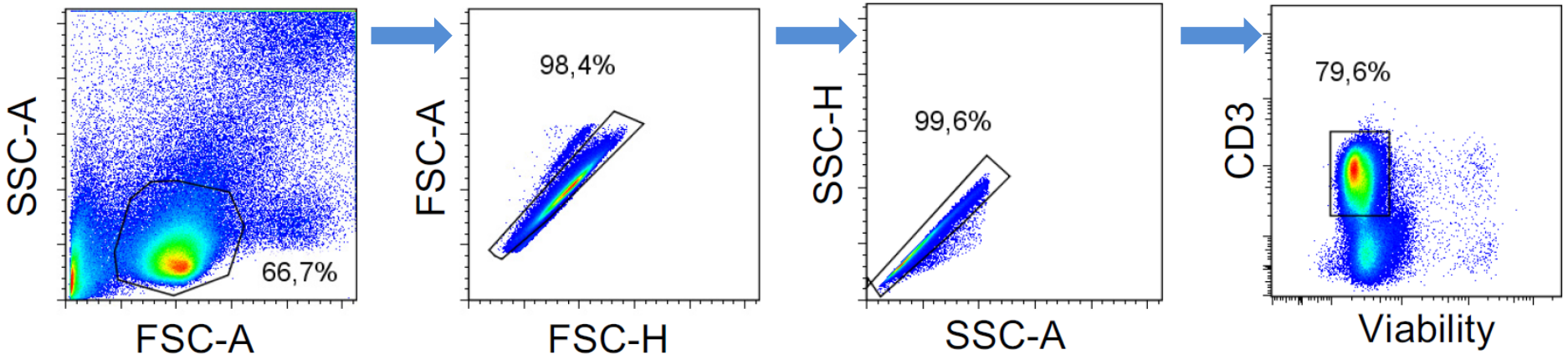


Antibody staining ***RNA/Flow assay***

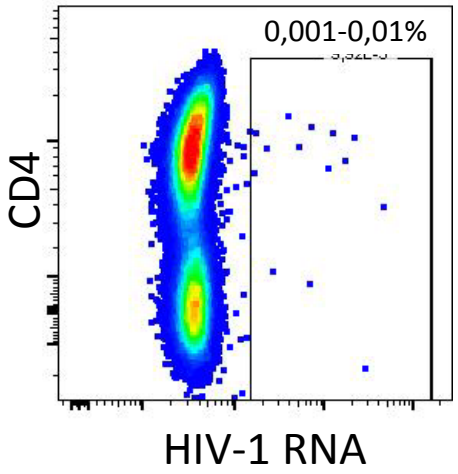
Grau J et al. mBio 2017

Detection of HIV-1 RNA in exvivo infected PBMCs

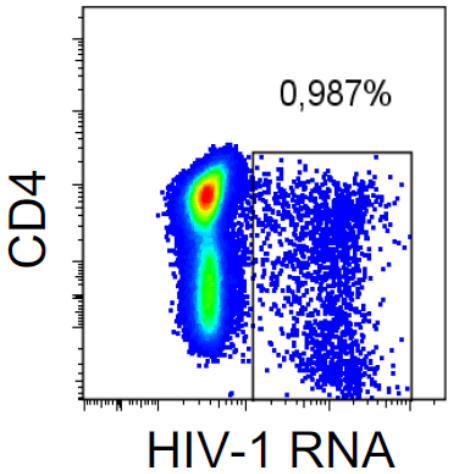
✓ **Gaiting strategy:**



HIV negative



HIV positive

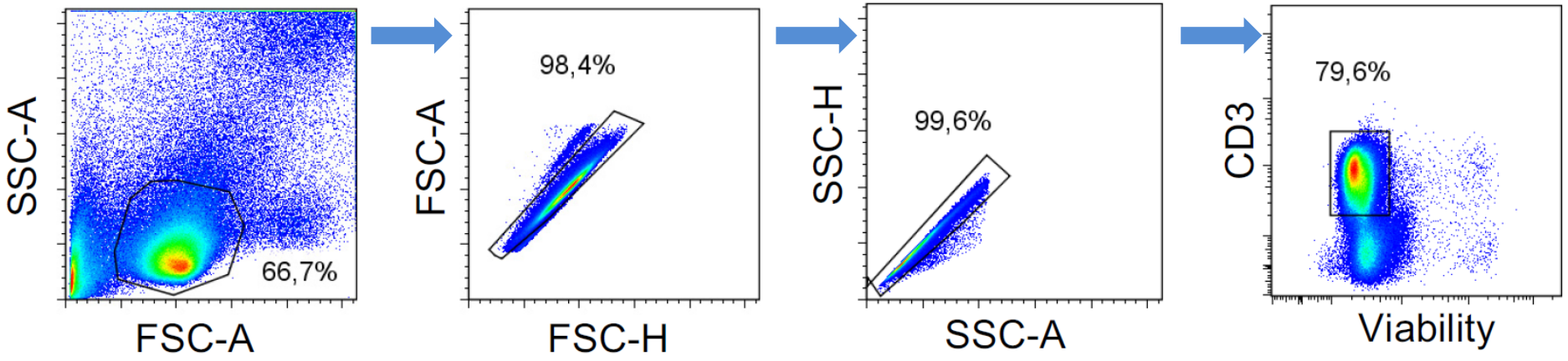


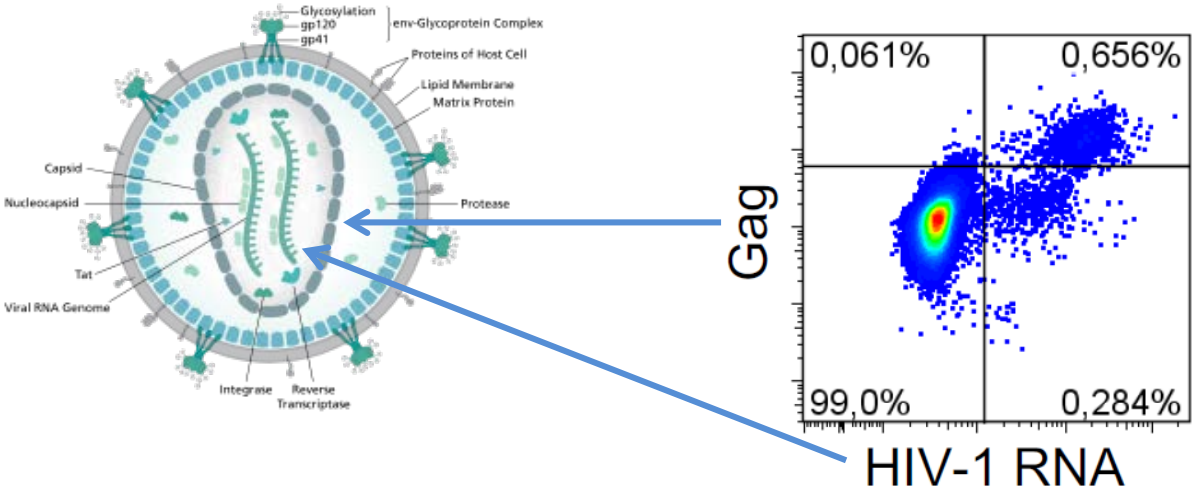
- Background highly dependent on the viability of the cells and probe used
- Gating Strategy very important to minimize background
- Background subtraction is required in each experiment using negative control data

Grau J et al. mBio 2017

Detection of HIV-1 RNA in exvivo infected PBMCs

✓ **Gaiting strategy:**





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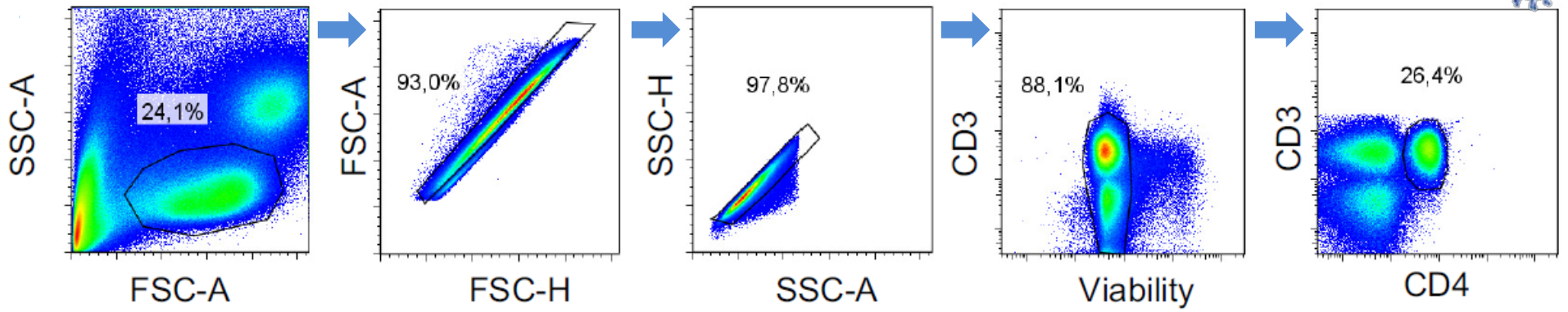
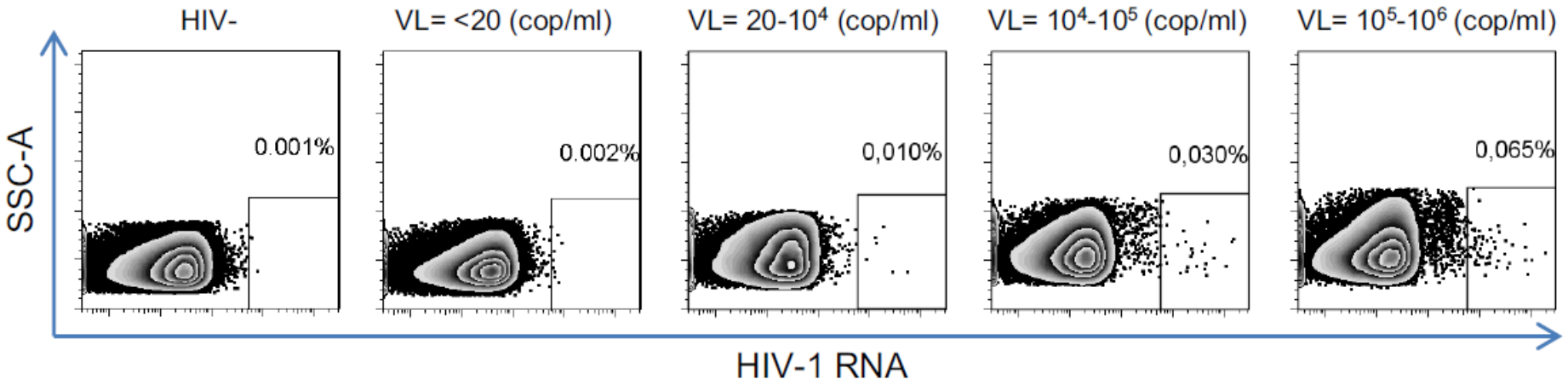
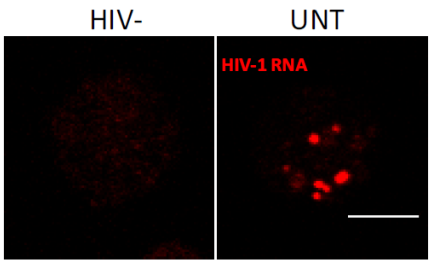
HIV-1 RNA detection in HIV-infected patients







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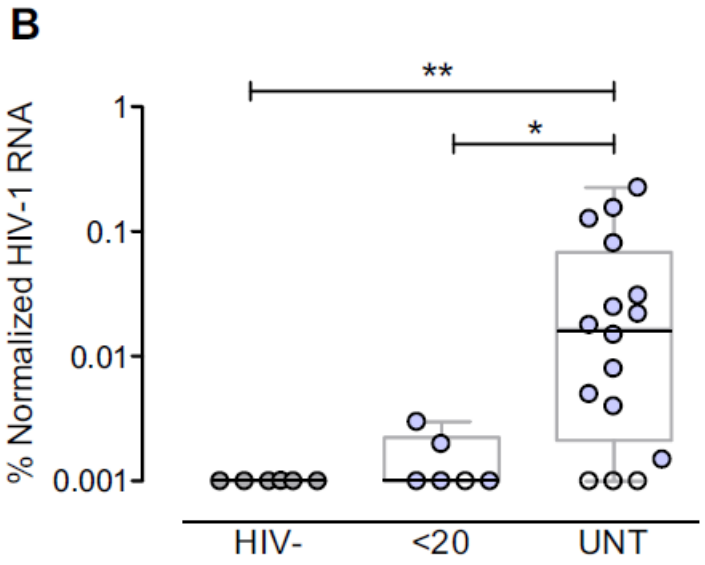
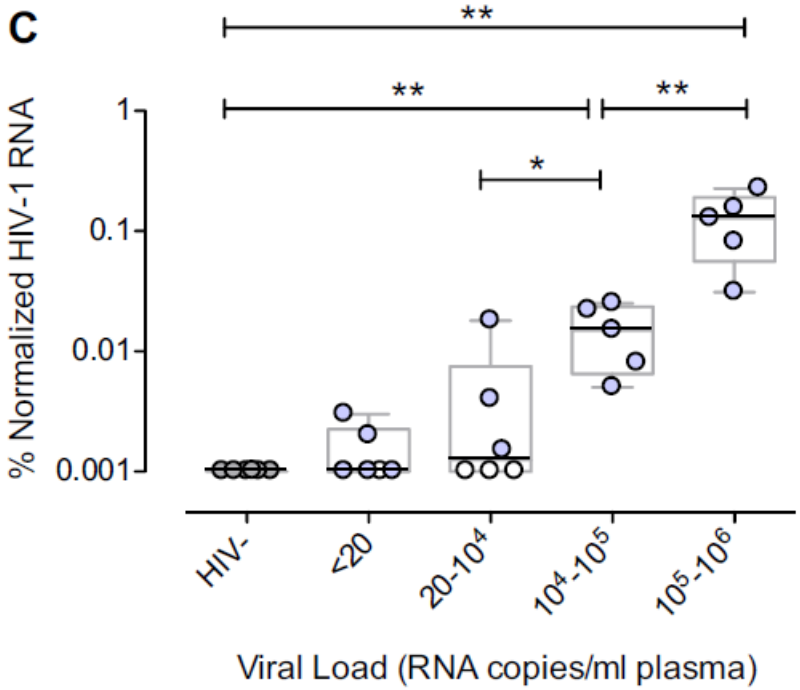
✓ Quantification of HIV-1 RNA signal:

% Normalized HIV-1 RNA = % HIV-1 RNA patient - % HIV-1 RNA uninfected donor





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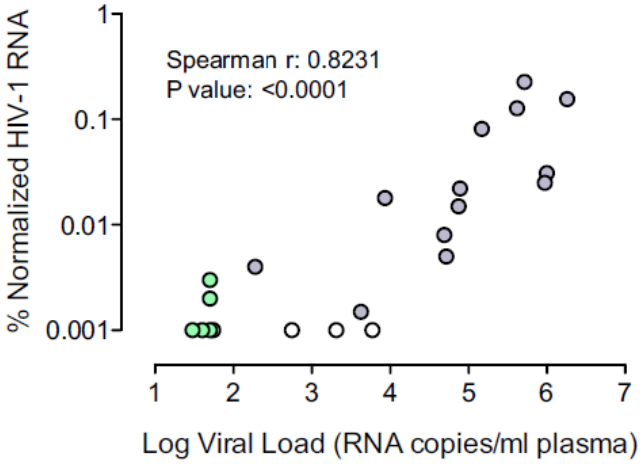
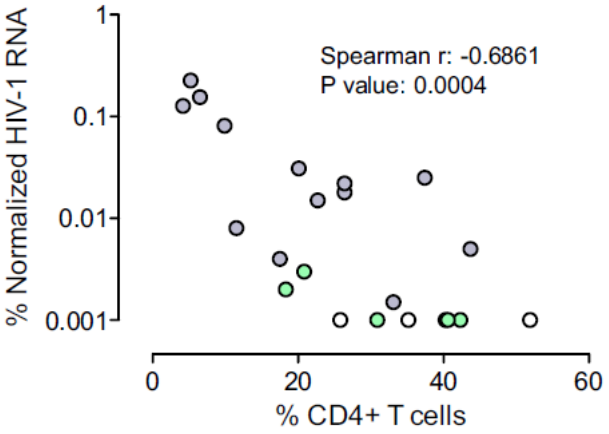
HIV-1 RNA detection in HIV-infected patients

✓ **Correlation of % HIV-1 RNA + cells:**





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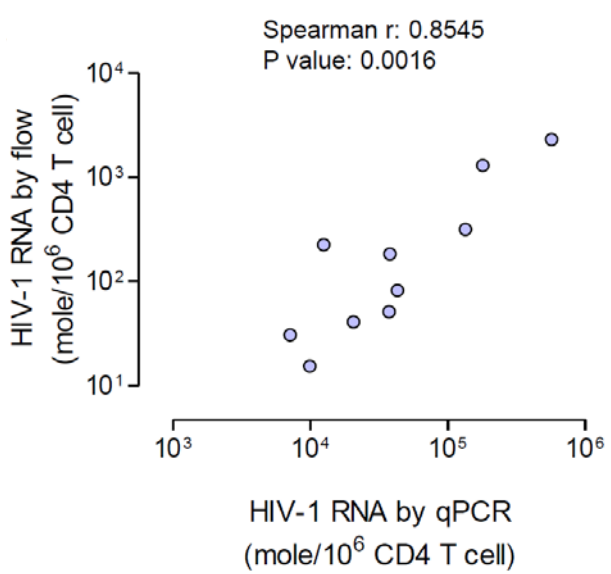
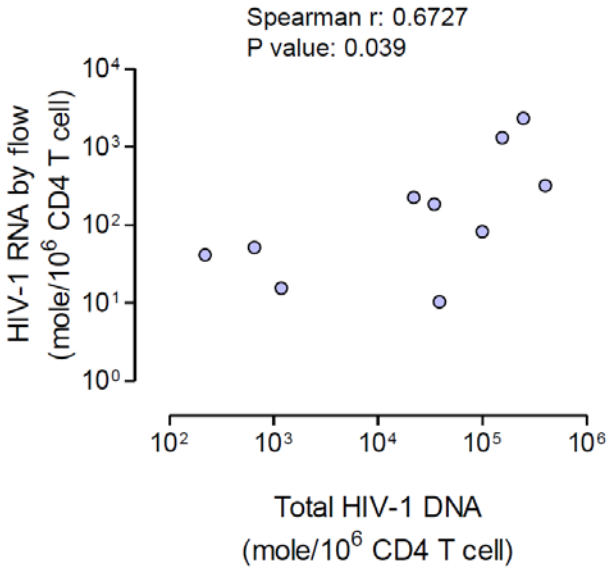
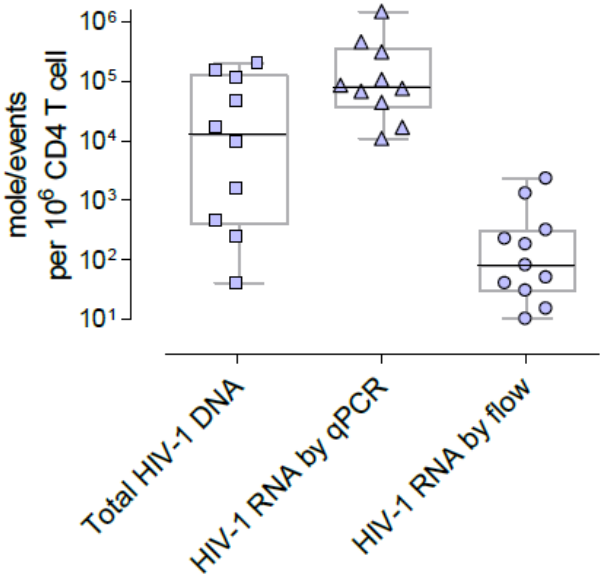
HIV-1 RNA detection in HIV-infected patients

✓ **Correlation of % HIV-1 RNA + cells:**







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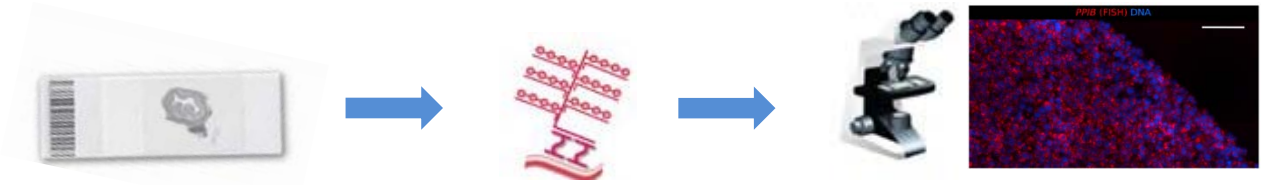
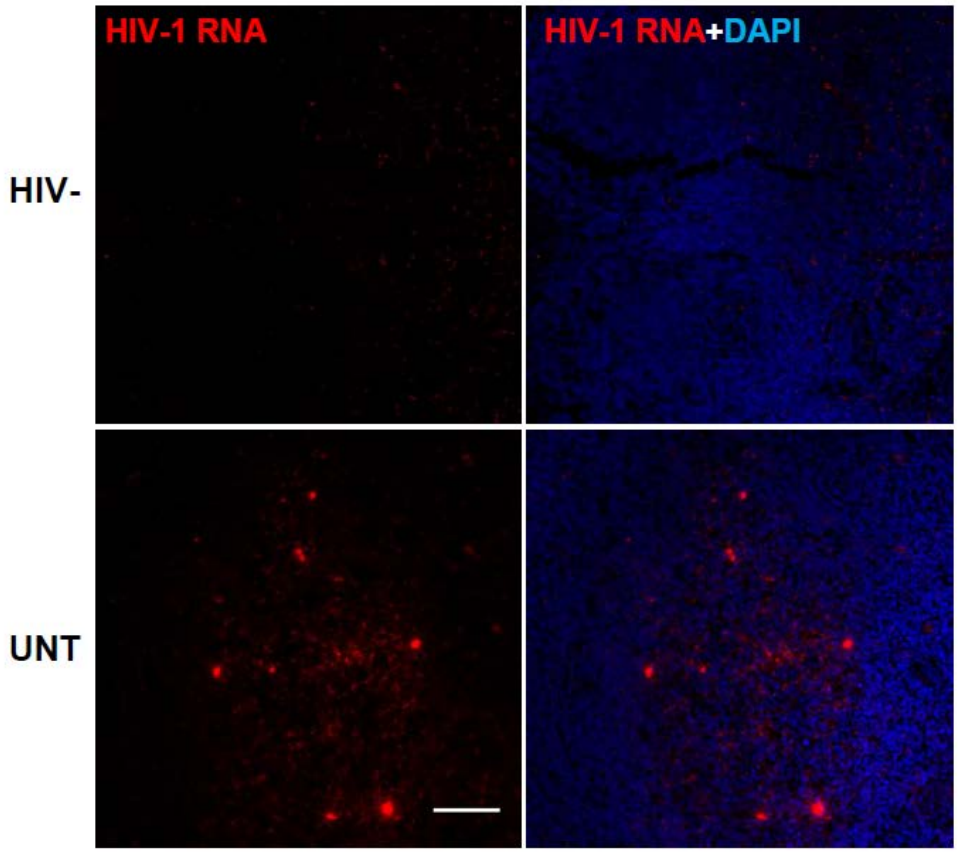
HIV-1 RNA detection in HIV-infected patients

✓ **Probes specificity:**



Lymph node slide | *HIV-1 RNA probe hibridization* | *Microscope analysis*



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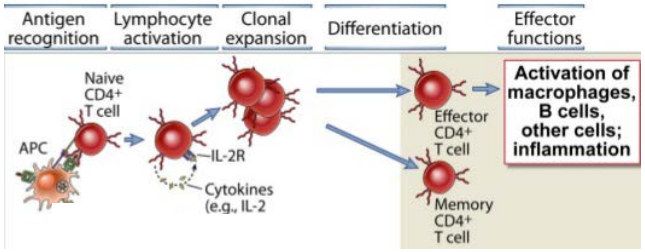
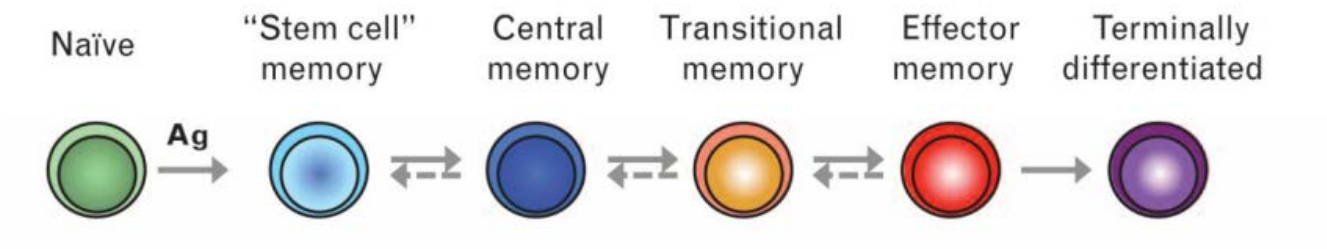
Subpopulations of CD4 lymphocytes

CD4 T cells activation



CD4 T memory subsets differentiation:



Lymphoid homing
Proliferation potential
Self-renewal
Multipotency

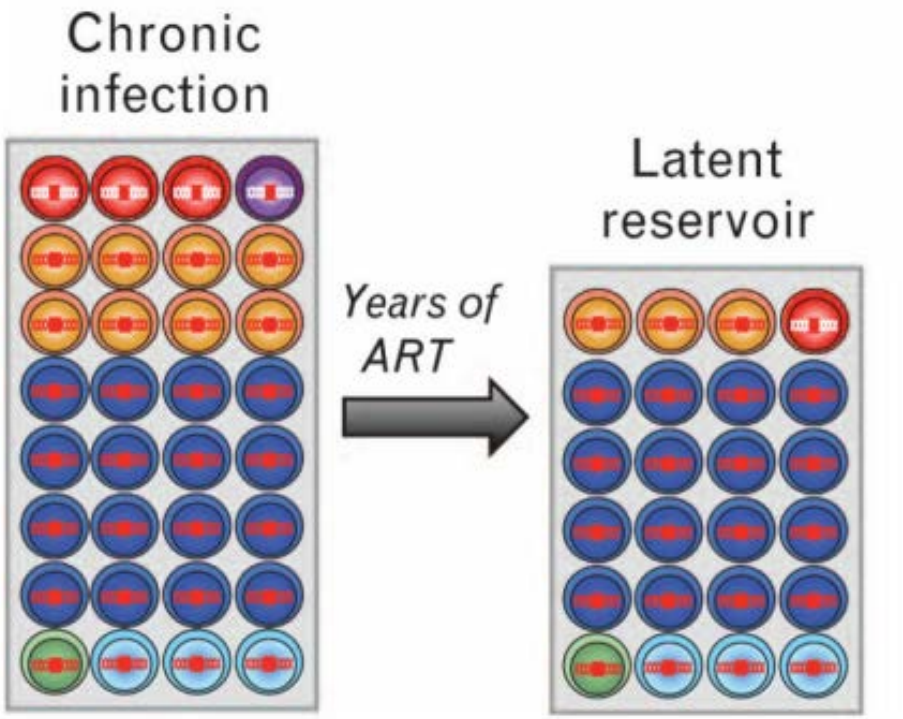
Peripheral homing
Effector function
Antigen dependence

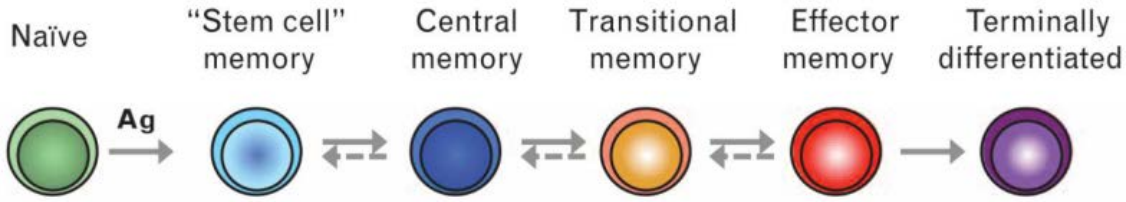
CD45RO	-	-	+	+	+	-
CCR7	+	+	+	-	-	-
CD27	+	+	+	+	-	-
CD95	-	+	+	+	+	+

Ananworanich, J., et. al., Curr Opin HIV AIDS (2015) Modified

Subpopulations of CD4 lymphocytes

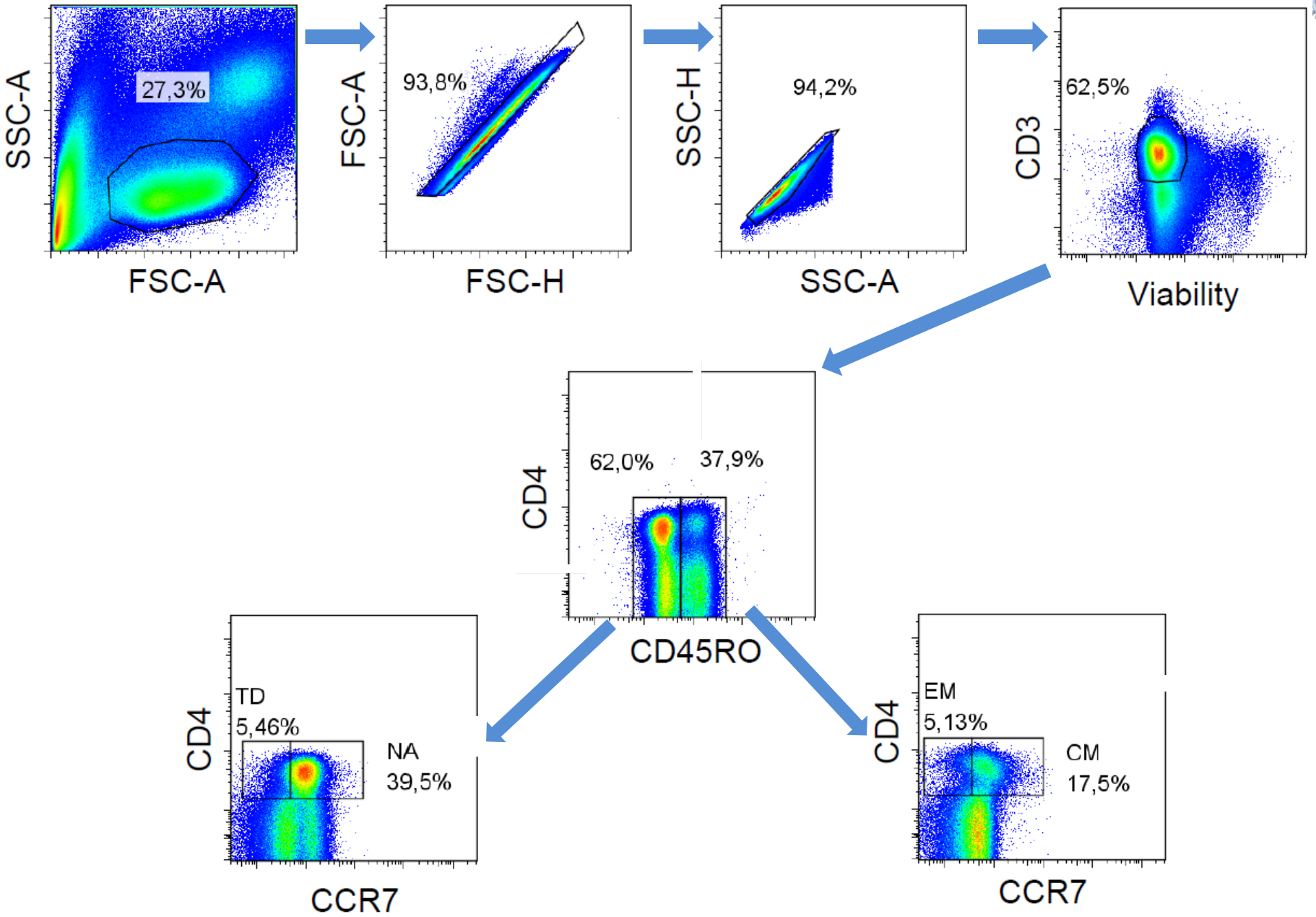
Viral persistence → long-lived cells (mainly T_{CM} and T_{SCM})





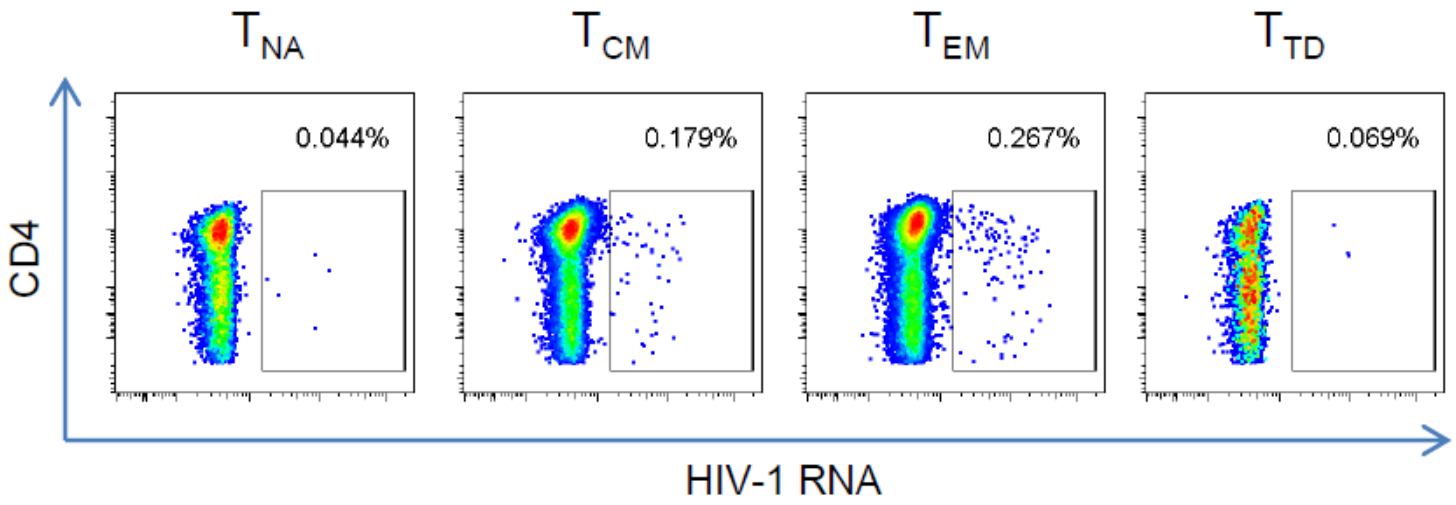
Ananworanich, J., et. al., Curr Opin HIV AIDS (2015)

Subpopulations of CD4 lymphocytes that express HIV-1 RNA

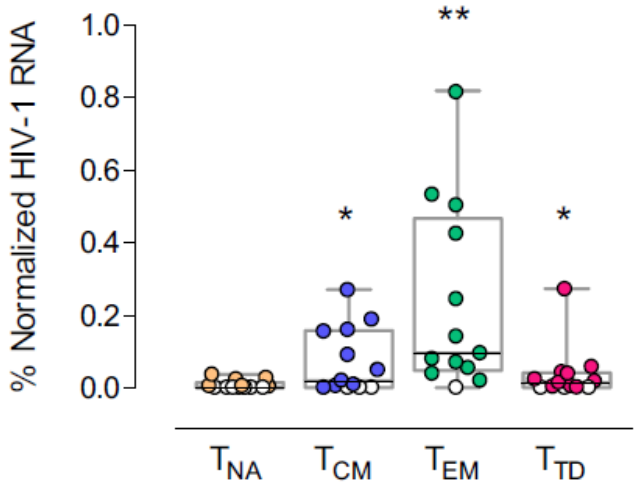


Grau J et al. mBio 2017

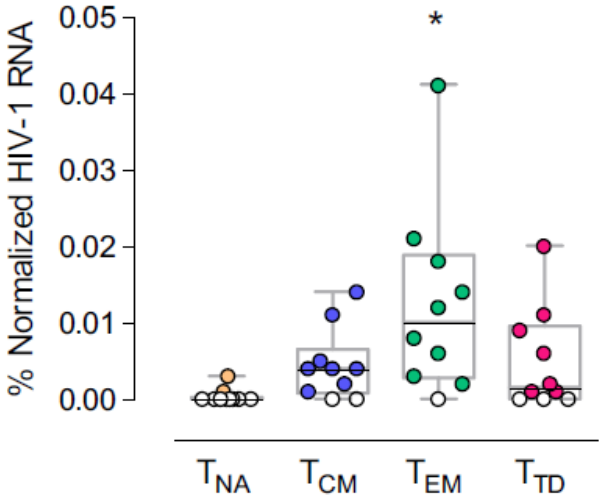
Subpopulations of CD4 lymphocytes that express HIV-1 RNA



Untreated patients

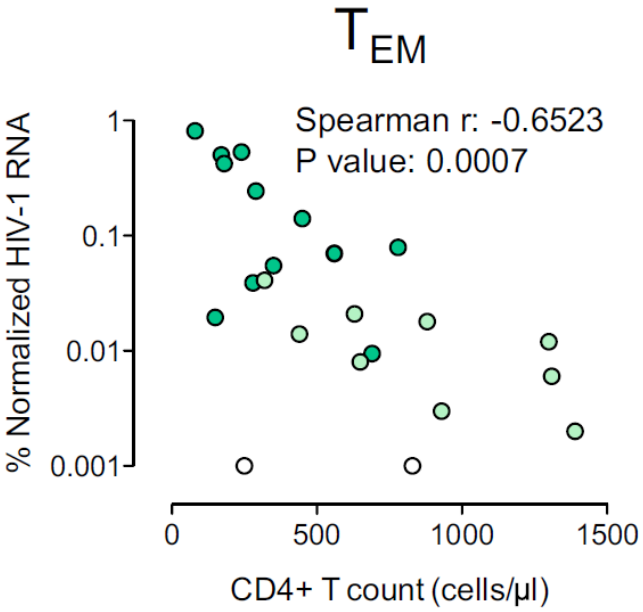


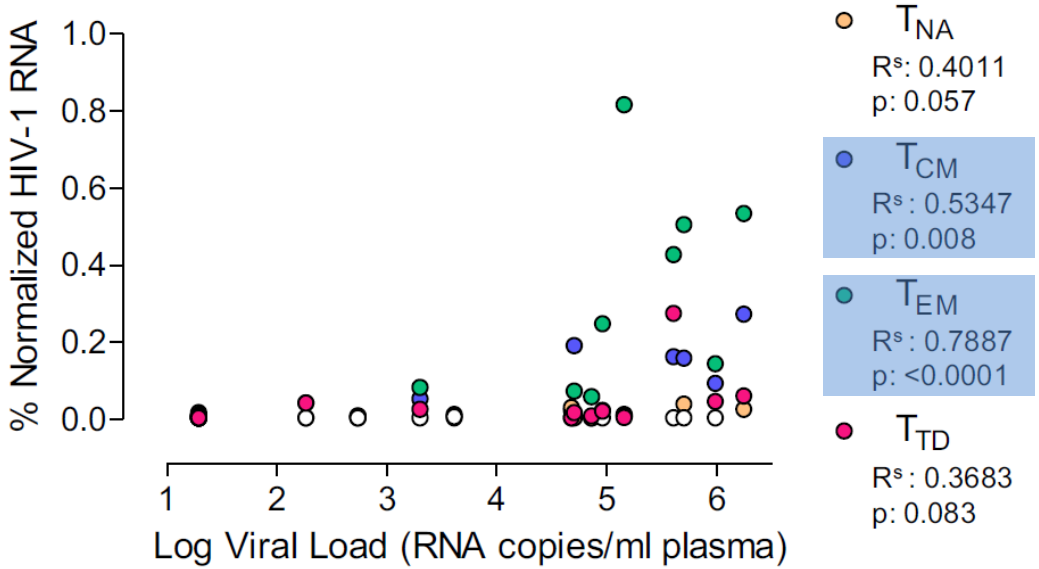
ART-treated patients



Grau J et al. mBio 2017

Subpopulations of CD4 lymphocytes that express HIV-1 RNA





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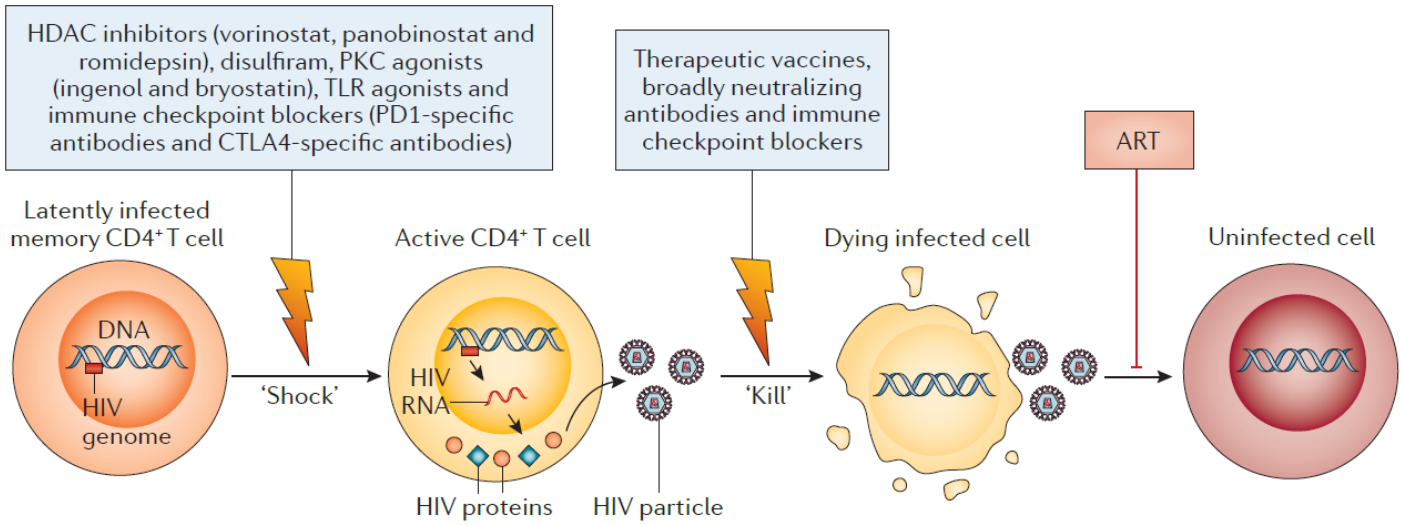
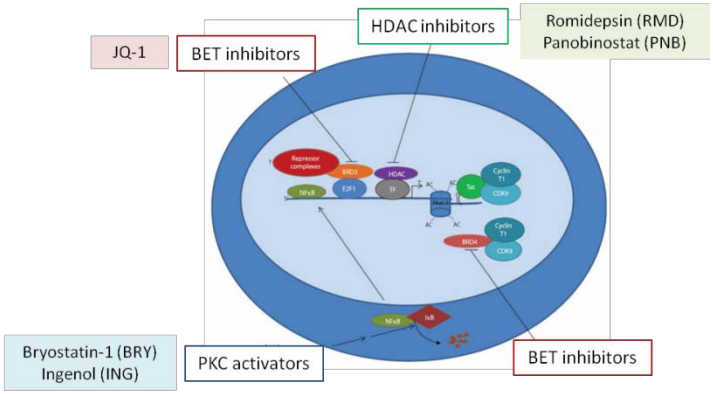
Shock and kill strategy

Shock and kill sratego:

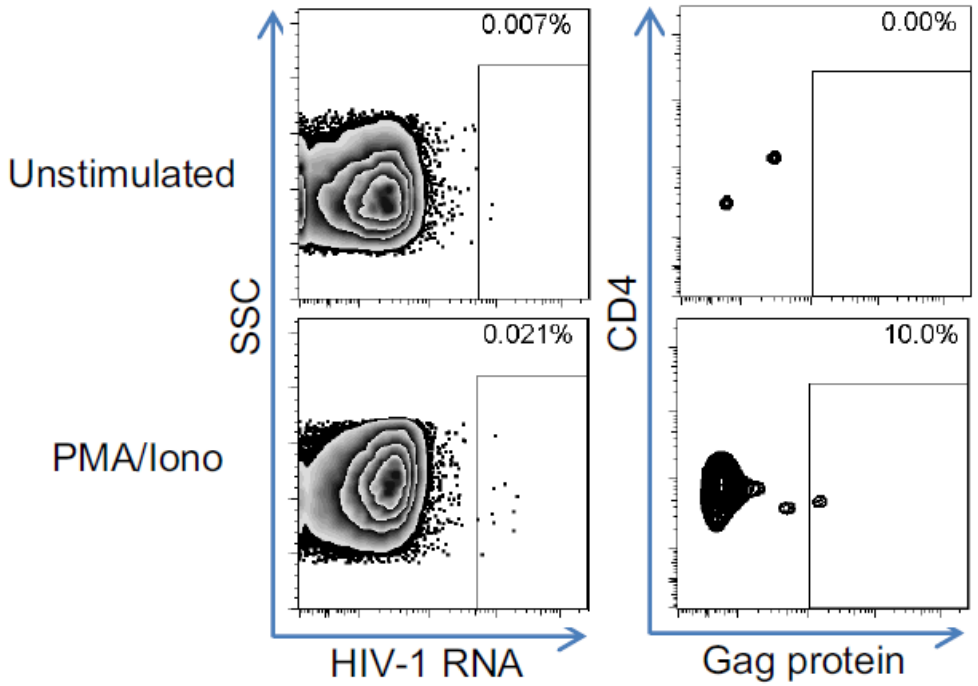

HDAC inhibitors (vorinostat, panobinostat and romidepsin), disulfiram, PKC agonists (ingenol and bryostatin), TLR agonists and immune checkpoint blockers (PD1-specific antibodies and CTLA4-specific antibodies)
Therapeutic vaccines, broadly neutralizing antibodies and immune checkpoint blockers
ART
Latently infected memory CD4+ T cell
Active CD4+ T cell
Dying infected cell
Uninfected cell
DNA
HIV genome
'Shock'
HIV RNA
'Kill'
HIV proteins
HIV particle

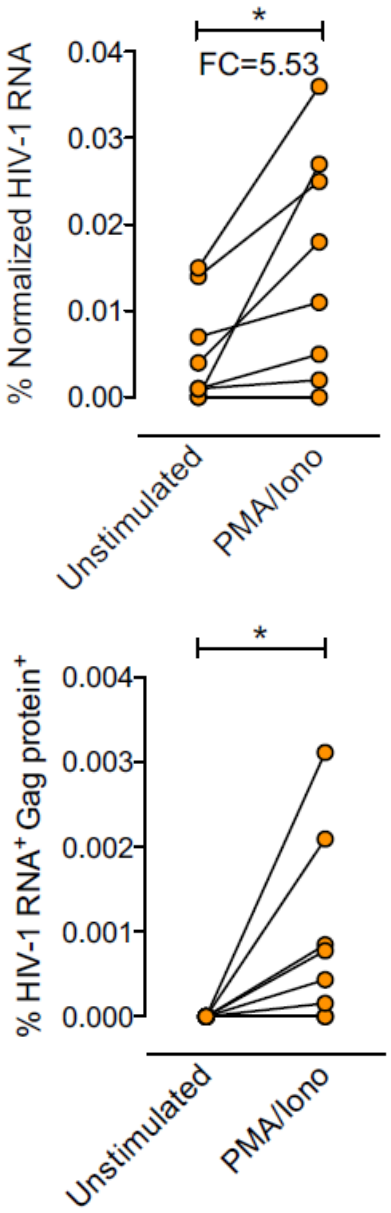
Latency reversal agents (LRA):



Deeks SG et. al. Nat Rev (2015) *Kim et al. Cell Host & Microbe (2018)*

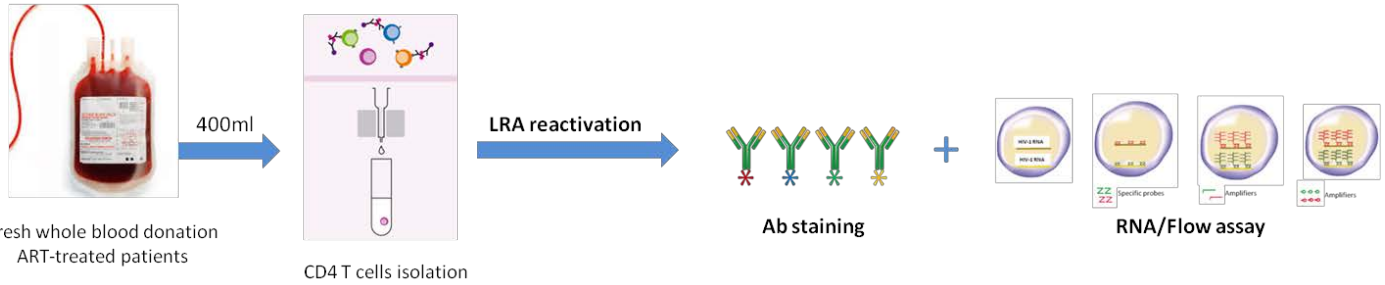
Reactivation by RNA/Flow assay



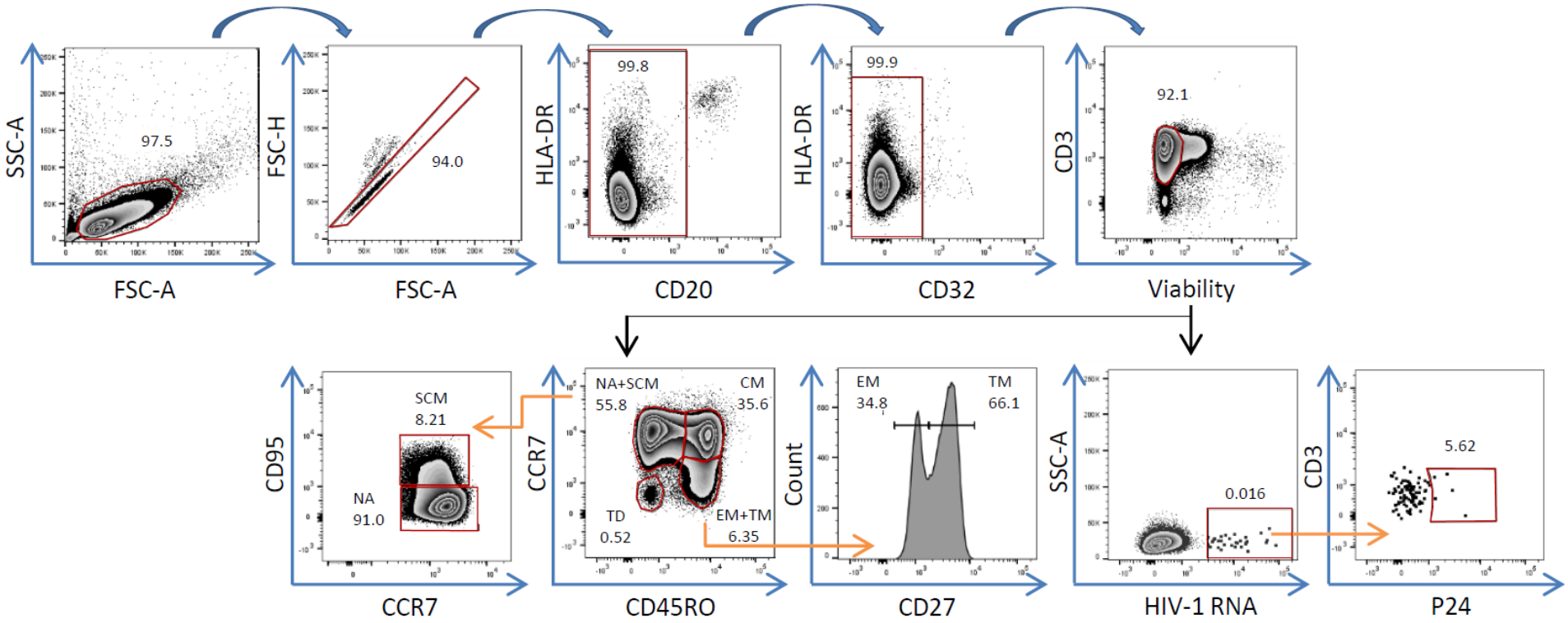


Grau J et al. mBio 2017

Reactivation by RNA/Flow assay

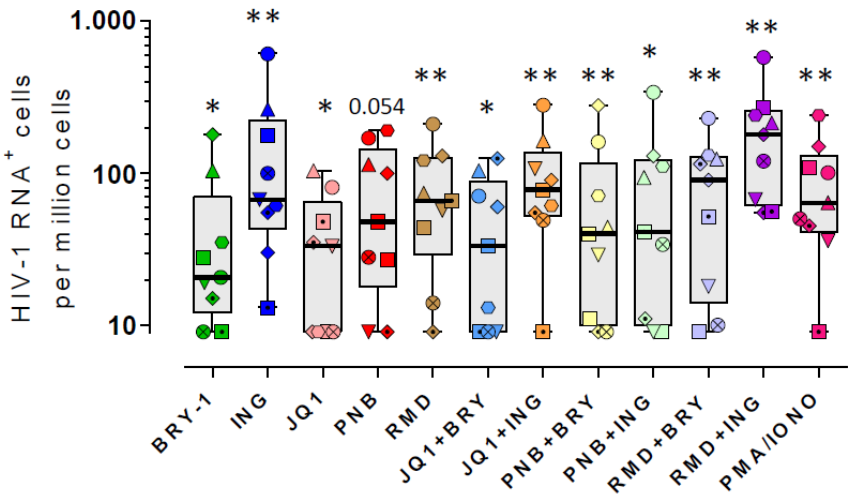


Gaiting strategy:

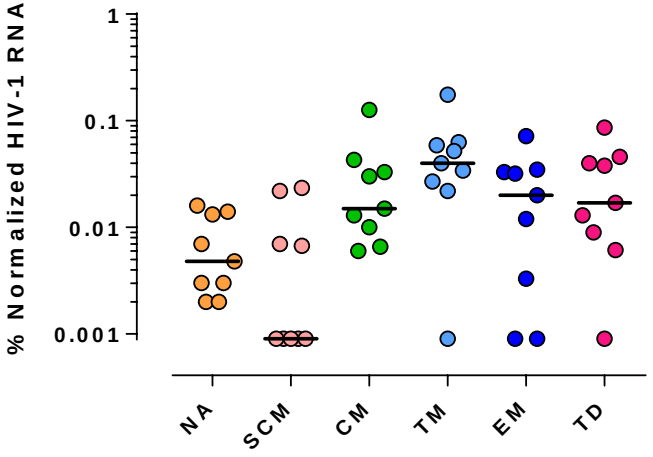


Reactivation by RNA/Flow assay

Total CD4 T cells:



CD4 T memory subsets:



Grau J et al. 2019 (submited)

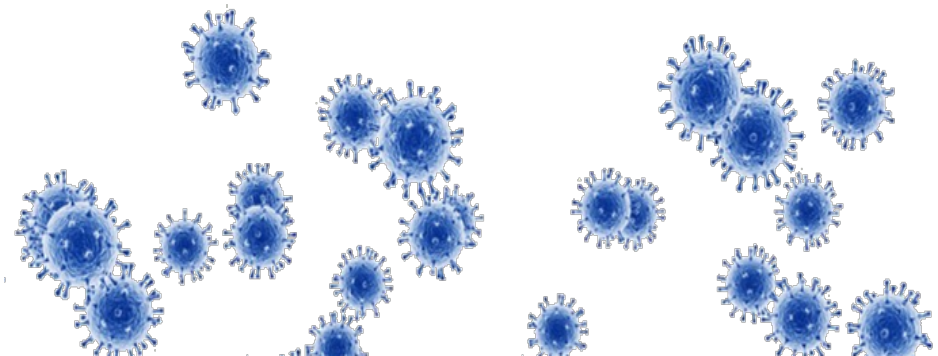
Summary

✓RNA/flow assay is a **valid method for detection of HIV-RNA expression** and viral production after *ex-vivo* infection of non-activated PBMCs.

✓With RNA/flow assay we are able to detect transcriptionally active cells in **primary samples** of HIV-infected patients.

✓We successfully detected different subpopulations of cells expressing HIV-RNA using the flow RNA/FISH protocol and we observed that the **effector memory T cell subset** as the main subset of cells expressing HIV.

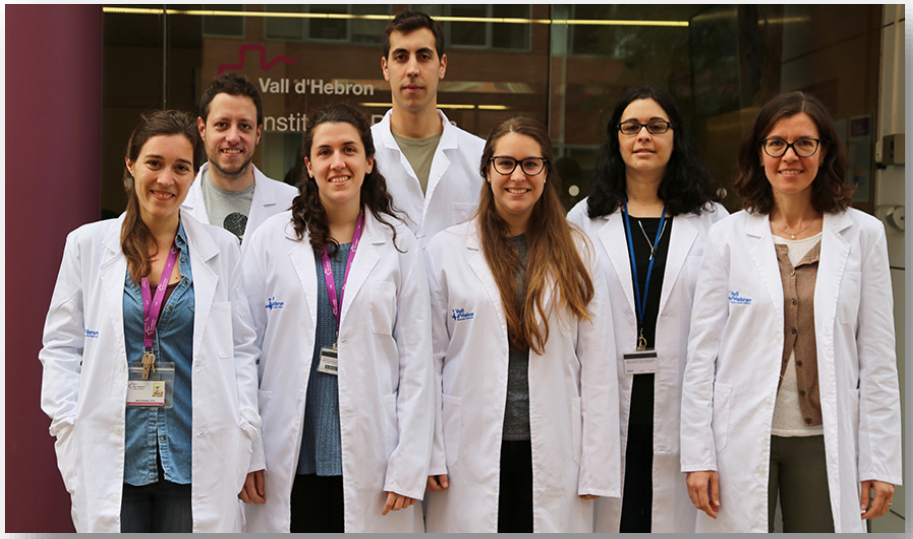
✓The synergistic **combination RMD and ING** seems to impact most $CD4^{+}$T cell subsets (in terms of HIV-RNA).



Acknowledgment

HIV Translational Research Group (VHIR)

Judith Grau
Antonio Astorga
Meritxell Genescà
Maria J. Buzón
Laura Luque
Jon Cantero



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Mario Martin

Banc de Sang i Teixits (Vall d'Hebron Hospital)

Jesus Fernandez
Marta Rodriguez

Bristol-Myers Squibb
GILEAD
NIH National Institutes of Health
For Women in Science FONDATION L'OREAL
Vall d'Hebron Hospital
RETIC-RIS Red Española de Investigación en SIDA (RIS)
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GOBIERNO DE ESPAÑA MINISTERIO DE ECONOMIA Y COMPETITIVIDAD